An investigation of the production of volatile organic compounds (VOCs) by *Trichoderma* spp. and their mode of action against *Serpula lacrymans*

Sonia Naomi Humphris

2003

An investigation of the production of volatile organic compounds (VOCs) by *Trichoderma* spp. and their mode of action against *Serpula lacrymans*

Sonia Naomi Humphris

A thesis submitted in partial fulfilment of the requirements of the University of Abertay Dundee for the degree of Doctor of Philosophy

This research was carried out in collaboration with the Scottish Crop Research Institute

February 2003

I certify that this thesis is the true and accurate version of the thesis approved by the examiners.

Signed .....[redacted]..... Date 12/6/03.....

Director of Studies

Declaration

I hereby declare that the following thesis is based on the results of investigations conducted by myself, and that this thesis is my own composition. This thesis has not, in whole or part, been previously presented for a higher degree. Work other than my own is clearly indicated in the text by reference to the relevant researchers or their publications.

Sonia Humphris.

Acknowledgements

I would like to express my sincere gratitude to my supervisors Dr Alan Bruce and Dr Ron Wheatley for their valuable help, advice and encouragement throughout this project. I would also like to thank Dr Eldridge Buultjens for his additional advice, his expertise and time with the SDS-PAGE.

I would also like to express my thanks to Dr Naoise Nunan for his help with the statistical analysis and Dr Nigel Deighton for his help with the ESI-MS and to other members of staff at SCRI for their advice.

Thanks to my friends Sheena, Debbie, Kirsty, Sandra and Susan for all their support and for all the laughs throughout my PhD.

Lastly I want to thank my mum, Jenny and sister, Louise for all their support (mainly financial) and without who I would never of made it through.

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ACN	acetononitrile
ANOVA	analysis of variance
APS	ammonium persulphate
CO_2	carbon dioxide
d	day
DTT	dithiothreitol
°C	degree Celsius
Fig	figure
GC-MS	gas chromatography-mass spectrometry
HCl	hydrochloric acid
KDa	kilo Daltons
MEA	malt extract agar
mA	milliamps
ml	millilitre
mM	millimolar
min	minute
MW	molecular weight
ng	nanogram
NH_4HCO_3	ammonium bicarbonate
%	percentage
PBS	phosphate buffered saline
PCA	principal component analysis
ppm	parts per million
rpm	revolutions per minute

SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
spp	species
TEMED	N,N,N′,N′-Tetramethylethylenediamine
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
Tris-HCl	Tris-hydrochloride
µg	microgram
µl	microlitre
u-p dH_2O	ultra pure distilled water
w/v	weight/volume
v/v	volume/volume
VOCs	volatile organic compounds

Abstract

All living organisms produce volatile compounds that can have a significant effect on the ecological balance within their community. In wood, volatile compounds can build up and become toxic to other resident microorganisms. The major aim of this study was to evaluate the production of volatile compounds by *Trichoderma* species and evaluate their importance as a mechanism of control of *Serpula lacrymans*. Volatile interactions between a range of *Trichoderma* isolates grown on 3 different media (malt extract, minimal media and sawdust) and 4 *S. lacrymans* isolates demonstrated that levels of inhibition were influenced by *Trichoderma* isolate, media type and sensitivity of the *S. lacrymans* strain. The growth inhibition of *S. lacrymans* consistently resulted in the production of yellow pigmentation in the *Serpula* mycelium. The greatest levels of inhibition were seen when the *Trichoderma* were grown on the malt extract media. However, significant levels of inhibition were still recorded when the *Trichoderma* spp. were grown on a minimal medium (with a carbon:nitrogen ratio similar to that found in Scots pine sawdust) and sawdust. The growth inhibition of *S. lacrymans* on the minimal media and sawdust is likely to provide a much more realistic guide to levels of inhibition that might be expected in the field. Gas chromatograph-mass spectrometry and associated principal component analysis of the *Trichoderma* VOCs resulted in the identification of 8 volatile compounds, mainly ketones, which were implicated as the active VOCs involved in the inhibition of *S. lacrymans*. The range and quantity of volatiles produced was dependent on the *Trichoderma* isolate and the media on which it was growing. If *Trichoderma* is to be successful as a remedial treatment of dry rot in buildings, the volatiles identified as active inhibitory compounds must also be produced by the potential biocontrol agent in wood. Therefore, compounds of particular interest were 3-octanone, 2-nonanone and 2-heptanone, as they were produced by the most

inhibitory treatment (*T. aureoviride* when grown on malt extract) but also produced on the sawdust.

After exposure of the *S. lacrymans* strains to the *Trichoderma* VOCs, the *Serpula* cultures were assayed for production of 3 enzymes, cellulase, tyrosinase and peroxidase. The results demonstrated that the *Trichoderma* VOCs did not affect cellulase production. Therefore, while there would be a decrease in cellulase production, due to the reduction in *S. lacrymans* biomass, caused by the inhibition of growth by the *Trichoderma* VOCs, *S. lacrymans* would still be capable of degrading cellulose. Tyrosinase production was not detected in any of the cultures of *S. lacrymans*. Since yellow pigmentation was detected in plates of *S. lacrymans* inhibited by the *Trichoderma* VOCs it is unlikely that tyrosinase plays a role in this pigmentation in *Serpula*. Increased peroxidase production was detected in all plates of *S. lacrymans* displaying yellow pigmentation, suggesting that increased peroxidase production is linked to increased pigmentation associated with a stress reaction in *S. lacrymans*.

SDS-PAGE analysis of protein production indicated changes in the protein profiles of *S. lacrymans* isolates when exposed to inhibitory VOCs from *Trichoderma*. One protein band in particular, at molecular weight 22.4 kDa, appeared to be closely linked to levels of growth inhibition with production of this protein completely inhibited by the VOCs from *T. aureoviride*, which gave the greatest levels of inhibition of the *S. lacrymans* isolates. The protein was unaffected by VOCs from *T. pseudokoningii*, which gave very little or no inhibition of growth of *S. lacrymans*, while VOCs from *T. viride* (which gave on average between 35-50% inhibition of growth) caused a marked reduction in synthesis of this protein. Analysis of this protein by electrospray ionisation did not, however, result in the identification of this protein. Removal of the antagonistic VOC

stress demonstrated that the inhibition of *S. lacrymans* growth was transient, with not only growth resuming but production of all proteins previously inhibited also resuming. This work has shown that VOC production by *Trichoderma* spp. can have a fundamental role in microbial ecology of ecosystems dominated by fungi and for the first time has shown both up and down regulation of protein production in *S. lacrymans* after exposure and subsequent removal of inhibitory *Trichoderma* VOCs.

CHAPTER 1: Literature Review

1.1. Wood

Wood is a remarkable material and has remained one of the most important renewable natural resources available to man. Wood has unique engineering properties and high strength per unit weight, which have confirmed its suitability as a constructural material for a range of end-uses. Timber is a convenient energy source and has provided material for shelter and other constructions, including material for making weapons, tools, utensils and furniture. In addition, wood provides a source of extractives for the chemical industry, is the basic raw material in the paper-making industry, is used in the textile industry and is also used in the manufacture of wood composites and panel products for the building industry (Eaton and Hale, 1993).

1.1.1. Wood decay

Wood in buildings is most commonly decayed by wood rotting basidiomycetes that divide into 2 subgroups, the white and brown rot fungi. The 2 groups have different strategies for decaying wood, in particular for dealing with the lignin that serves as a chemical and physical barrier to enzymic degradation of wood polysaccharides. During brown rot decay, hemicelluloses are rapidly utilized and the degree of polymerisation of the wood cellulose decreases quickly, which has severe consequences with respect to wood strength properties. In the early stages of decay caused by brown rot fungi there is considerable reduction in the mechanical strength of the wood but little commensurate weight loss. Brown rot fungi strongly decrease the methoxyl content of lignin in wood with the result that lignin is only modified and slightly depleted resulting in the reddish to dark brown appearance of the decayed wood (Kirk, 1971). During the later stages of decay, dry wood commonly shows deep cross-cracking, due to shrinkage caused by loss of wood cell wall carbohydrates and the wood may also show longitudinal cracks. White rot fungi, however, cause a gradual decrease in the degree of

polymerisation of the cellulose and produce phenol-oxidases that enable them to degrade lignin extensively resulting in wood with a bleached appearance (Kirk, 1971). The surface of white rotted wood is often softened and may become fibrous along the grain due to separation of the woody cells along the middle lamellae and in some cases the wood may become pulpy. Strength loss in wood decayed by white rot fungi is proportional to the loss of cellulose. Some white rot and brown rot fungi can produce smaller regions or pockets of decay surrounded by sound wood during the early stages of decay (Eaton and Hale, 1993).

Different terms have been used to categorise decay since the recognition that fungi cause decay of wood in natural and man-made environments. Brown rot and white rot are currently in widespread use today. However, many building surveyors will define fungal decay types as dry rot or wet rot. Dry rot fungi require a minimum level of 20% moisture in wood for colonisation but, in general, contents of 30-50% allow growth (Cartwright and Findlay, 1958) whereas wet rot fungi can only start to colonize wood at 26% moisture content. The term dry rot is an appropriate way of describing the wood in the advanced stages of decay, as the modified lignin residue can often appear dry and crumbly to the touch. In the final stages of decay caused by fungi that require wood to be comparatively wet for them to initiate or continue attack, the infected area is often itself wet hence the term wet rot. By far the most important fungus destructive to wood in service in buildings is the dry rot fungus *Serpula lacrymans*, which will attack drier wood than most fungi but not below 20 % (Desch and Dinwoodie, 1996).

1.2. *Serpula lacrymans*

S. lacrymans is one of the best known brown rot basidiomycetes and is the most destructive wood decay fungus of buildings in temperate regions outwith the USA (Jennings and Bravery, 1991). *Serpula* does not usually occur in wetter conditions, in this situation a wet rot fungus such as *Coniophora puteana* occurs instead. *S. lacrymans* is the causative organism of dry rot of timber and together with 2 wet rot basidiomycetes (*Coniophora puteana* and *Fibroporia vaillantii*), is responsible for 95% of decay of building timber in Britain (Coggins, 1980). This fungus is probably the most economically important fungus occurring in buildings in the UK and Europe. *Serpula* causes millions of pounds worth of damage annually. For example, in the UK the estimated cost of rectifying damage caused by *S. lacrymans* is at least £150 million per annum (Jennings and Bravery, 1991).

1.2.1. Occurrence of *S. lacrymans*

S. lacrymans is widespread in timber in buildings in most cool temperate regions, such as Northern and Central Europe, Australia, and Japan. Due to its sensitivity to heat, *S. lacrymans* is usually exclusively found within timber in buildings, but it was reported growing in the wild on coniferous stumps and logs at 5 different locations in the Himalayas (Bagchee, 1954), forests of the Czech republic (Soukup, 1979) and the Californian mountains (Harmsen, 1960).

The morphological identification of *S. lacrymans* is often confused with that of *S. himantiodes* a close relative, which has been found in the wild in temperate regions. It is generally believed that *Serpula* is found in buildings in temperate regions because the built environment is climatically similar to the Himalayan foothills and the fungus is unusually sensitive to changes in the environmental temperature. White *et al.* (1997)

speculated that colonisation of *Serpula* in the wild might have been restricted by competitor organisms growing in more intact wood and timber. *Serpul*a may only grow in nature under conditions achieved in well-decayed timber due to the higher moisture contents attained in decomposing wood or because of metabolites released by previous colonisers. White *et al.* (1997) confirmed the first successful culturing and confirmation of identity of *S. lacrymans* derived from "basidiomes and mycelia growing in the wild", on well decayed coniferous wood of the Himalayas.

Figure 1.1. *Serpula lacrymans* growing in the Himalayas on a well decayed log and extending on to soil. Reprinted with kind permission of Dr Nia White.

1.2.2. Appearance of *S. lacrymans* on wood

Hyphae are not necessarily seen on the surface of heavily decayed wood and growth and levels of decay can be extensive before the fungus becomes evident. Some species produce prominent strands and under conditions of stagnant air flow and high relative humidity a fluffy mycelium can be seen. Under moist conditions development of the mycelium on the wood surface results in a white cotton wool textured mycelium. Under less humid conditions and when the mycelium has aged, a skin of silvery grey, silky mycelium is formed and tinges of yellow and lilac are often seen. At the tips of the hyphae droplets of water are produced which gives rise to the name 'lacrymans', meaning in Latin, tears. The name 'Serpula' in Latin means to creep. *S. lacrymans* can be readily confirmed by the presence of fruiting bodies, which are often formed in buildings. Once established within a building, dormant or growing hyphae can remain hidden within the structure and re-emerge to cause fresh outbreaks of dry rot (Eaton and Hale, 1993).

The initiation of attack is always associated with the ingress of water into buildings and an increase in the moisture content of wood. *Serpula* is particularly sensitive to environmental extremes and will not grow in waterlogged wood or at temperatures above 26°C or below 3°C, with the optimum temperature for growth being 21-23°C. *Serpula* can colonise and begin to decay timber with a moisture content of 20%, although the optimum for its decay activities is 30-40%. Favourable conditions in a building for growth are high relative humidity, stagnation of air currents and low light levels. On the basis of these narrow physiological requirements it is surprising that *Serpula* is so successful at causing so much damage to timber in buildings and is so difficult to eradicate completely from infected timber, brickwork and masonry. The

potency of this fungus from a physiological point of view is not just its ability to attack timber but its ability to spread through a building from infected timber for considerable distances over non-nutritional surfaces and transport water and nutrients from moist wood to dry wood in new locations (Nuss *et al.*, 1991). The distances over non-nutrient surfaces that can be traversed are considerable and can be up to 12 meters (Savory, 1964).

Figure 1.2. Severe dry rot attack on floor joists. Reprinted with kind permission of Dr Nia White.

1.2.3. Mycelium development in *S. lacrymans*

Serpula produces 2 morphologically and physiologically different types of undifferentiated mycelium: one has the potential to produce strands and the other has the potential to transform into a spore producing fruit-body. Environmental conditions, especially humidity, may play a major role in the triggering of the initiation to form either strands or fruit-bodies or it may be genetically determined (Nuss *et al.*, 1991). Cymorek and Hegarty (1986) found that a wheatflour/malt extract medium or an oat grain medium supported good fructification in the laboratory at a temperature of 12°C. They also discovered that light is an important factor in stimulating fruiting body formation as no fructification occurred in complete darkness. However, no studies have produced definitive information on the requirement of light for fructification since the precise influence of light or darkness is difficult to monitor under field conditions. Fruit-body formation rarely occurs spontaneously in the laboratory but in the field it occurs when food material is exhausted. The fruiting bodies do not have any distinct shape and size and usually form in exposed areas where spores can be widely disseminated into the environment (Hegarty, 1991).

Strands are a composite of 3 mycelial types and are initiated approximately 1cm behind the colony margin in a process in which the wide main, empty looking vessel hyphae start to become ensheathed by thick walled, impermeable fibre hyphae. Both of these are bound together with narrower metabolically active tendril hyphae, which result in adhesion between the 3 hyphal types. Fungi whose sole nutrient source is wood need to be efficient at extracting and conserving nitrogen, as wood is carbon rich but nitrogen poor. Strands are thought to have 2 functions and roles for *S. lacrymans*, the first one being a means of conserving nitrogen. The young mycelium is able to extract the

nitrogen in wood and concentrate it, which is demonstrated by the fact that nitrogen derived from wood is accumulated in the mycelium to levels well above those in surrounding wood (Watkinson, 1984). As a conservation measure the second function involves recycling nitrogen by acting as a connection between autolysing mycelium on an exhausted substrate and mycelium in a secondary substrate that is undergoing colonisation (Watkinson, 1975). In the field situation thick walled strand material is formed which acts as a link to conduct food and water to the colonizing mycelium from the regions of substrate utilization. The capacity of *Serpula* to produce copious droplets of liquid is strong circumstantial evidence for the movement of water through the mycelium (Jennings, 1991). This enables the mycelial form of the fungus to traverse barriers, such as bricks and mortar, so that new timber may be colonized whilst the established mycelium is still within an exploitable timber substrate.

Watkinson, (1971a, 1984) has argued that colony differentiation of *Serpula* into strands is closely related to its nutrient economy. Strands are formed either when mycelium traverses a non-nutrient substratum or when growing on media exhausted of nutrients. However, the spread of mycelium may not be limited by the total amounts of nitrogen but by the ability of the mycelium to translocate the available nitrogen. In addition to translocation of nutrients, expandable pools of free intracellular amino acids (Venables and Watkinson, 1989) and nitrogen storage proteins decomposed in the absence of external nitrogen sources all assist in the maintenance of a nitrogen balance during times of stress.

1.2.4. Action of *Serpula* decay

The features of brown rot decay have led to the implication that the process of cellulose breakdown involves two steps. The first step is when the crystalline state of cellulose is

rendered more susceptible to attack and has been termed amorphogenesis (Coughlan, 1985). Initially it was thought that as with the second stage of cellulose breakdown, it is enzymatic (Mandels and Reese, 1961). It is now commonly believed, however, that a low molecular weight compound, which can more readily diffuse into wood because enzymes are too large to penetrate wood to reach the cellulose, brings about the process. Haliwell (1965) was the first to propose the possible existence of a non-enzymatic cellulytic system involving hydrogen peroxide (H_2O_2) and iron. Haliwell reported that the action of hydrogen peroxide and iron on crystalline cellulose mimicked the early stages of degradation of brown rot fungi. Koenigs (1974a,b) suggested that brown rot fungi oxidise cellulose via an H_2O_2/Fe^{2+} system as brown-rot fungi copiously produce extracellular hydrogen peroxide and that wood contains enough iron to make Haliwell's hypothesis possible.

During the early stages of decay, large quantities of oxalic acid are produced by the hyphal tips of *S. lacrymans*, which makes the wood highly acidic, with dry rotted wood being about pH 3. It is commonly known that hemicellulose is hydrolysed in strong acid and Bech-Anderson (1987) postulated that oxalic acid excreted by brown rot fungi might be the initial compound to attack the cell wall. In this way, cellulose-degrading enzymes would have free access to cellulose, which in turn would break down into water-soluble sugars than can be absorbed by the fungi. Oxalic acid is a low molecular weight compound that as such can penetrate the cell wall. Savory (1980) found almost complete degradation of the calcium components of plaster blocks attached to pieces of wood which had been inoculated with *S. lacrymans*. Bech-Anderson (1985) noted that dry rot attacks in Copenhagen were never more than 1 meter away from a source of calcium. This author has concluded that *S. lacrymans* is found close to sources of calcium in order to neutralise the very acid conditions generated as a result of the

production of oxalic acid. Oxalic acid along with calcium forms a water insoluble salt, calcium oxalate, and gives the fungus the opportunity to regulate the acidity of the substrate and thereby create an optimum pH in the environment for growth. The large quantities of calcium oxalate found in brown rotted wood and in the humus layer of the soil are thought to be the remains of the initial phases of wood decay. Green *et al.* (1991) have shown that the structural strength of timber is reduced after treatment with oxalic acid before a weight loss can be detected and that the amorphous cellulose is attacked at the same time as the hemicellulose, which concurs with the effects of brown rot decay.

Paajanen and Ritschkoff (1992) however, have proposed that it is not calcium in stone based buildings material which promotes the decay capacity of *Serpula* but rather the iron which is utilized in a H_2O_2/Fe^{2+} reaction after being solubilised by the acid. Palfreyman *et al.* (1996) designed a study to confirm if calcium was involved in decay caused by *S. lacrymans* and if so whether the role of calcium could be that suggested by Bech-Anderson. They found that increasing calcium nitrate concentration promoted decay of wood blocks and increased *Serpula* growth rates. *S. lacrymans* also acidified the various media used, but surface pH profiles indicated that the organism was able to buffer acid production to a certain extent when calcium nitrate, or other divalent ions were available. The results demonstrated that calcium was required for the decay process of *Serpula* in accordance with Bech-Anderson but the results also indicated that *Serpula* required calcium even at the start of decay when there was little accumulation of oxalic acid. In addition, the presence of calcium at the start of the decay process did not inhibit the decay, suggesting there may be more than one role for calcium in *Serpula* wood degradation.

The physiology of *Serpula* has been extensively studied. Investigations into strain characteristics (Wazny and Thornton, 1989), the wood rotting ability of strains (Thornton and Wazny, 1986), comparisons between growth rates and decay ability (Watkinson *et al.*, 1981; Thornton, 1985; Bravery and Grant, 1985; Thornton and McConalogue, 1990; Wazny and Thornton, 1991), translocation (Watkinson 1971b; Brownlee and Jennings, 1981), production and location of proteinases (Venables and Watkinson, 1989), utilisation of soil components (Doi and Togashi, 1990) and toxicant tolerance studies (Schmidt and Moreth-Kebernik, 1989a). The requirements for better detection and control systems of *S. lacrymans* which rely on a more thorough understanding of the physiology of the organism means that molecular biology is also beginning to have an impact on *S. lacrymans* research.

1.2.5. Application of molecular biology to *S. lacrymans* detection/identification

Application of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for protein analysis of *S. lacrymans* has revealed very little variation between building isolates from Europe or Australia, with only one isolate (BF050) displaying any differences from the standard culture strain *S. lacrymans* FPRL 12C (Vigrow, 1992). However, there were significant molecular differences between *S. lacrymans* and other basidiomycete fungi and SDS-PAGE has therefore been proposed as a general identification tool for *S. lacrymans* (Schmidt and Moreth-Kebernik, 1989b; Palfreyman *et al.*, 1991; Vigrow, 1992). The potential use of molecular methods to detect and identify *S. lacrymans* from building material without the need for prior isolation and culturing has been investigated. The detection of *S. lacrymans* before any structural damage has occurred to a building is desirable from an economic point of view. Many workers have examined the potential of using antigenic differences to achieve this. Vigrow (1991a) produced a polyclonal antiserum against *S. lacrymans* 12C and used

this in conjunction with western blotting to try to detect *S. lacrymans* without the need to isolate and culture the fungus from the timber. All isolates gave identical antigen profiles, which were different from other decay fungi. Identification of S*erpula* from artificially infected wood blocks was possible but the antiserum also reacted with a wide range of different fungi and the wood products interfered with the banding patterns. In addition, unless the samples were taken from obviously live material, the overall antigens detected became too small to allow accurate identification. This would be a problem in the field if the only samples tested were from part of a colony that was no longer viable. Next Toft, (1993) produced a polyclonal antiserum against an antigen from a whole mycelial extract of *S. lacrymans* with a molecular weight of about 23 kDa via immunoblotting. It was specific towards this species and reacted with this antigen in protein extracts from all investigated strains of S*erpula* under denaturing conditions. This was only achieved with S*erpula* cultured on malt extract agar and not isolated from wood. Burge *et al.* (1994) looked at the possibility of using monoclonal antibodies (Mab) to provide a better basis for detection. Assays using monoclonal antibodies provide a higher degree of specificity and are relatively simple to perform and the potential to develop kits suitable for mass production and low cost commercialisation is well established. The Mab gave no cross-reaction with any other fungi, and all the *Serpula* isolates gave positive results. There was no interference between the antigen and the wood particles. However, in all cases the fungus was in an active state until its preparation for assay. Dead material showed no interaction with the Mab. This suggests that the Mab specificity was based on a non-structural component of the hyphae and possibly an enzyme, which though not destroyed during sample preparation was not present in non-viable mycelium.

Any identification method based on proteins incurs problems due to differential expression of proteins, during for example the life cycle of *S. lacrymans*, or changes in protein expression during environmental stresses. Vigrow *et al.* (1991a) demonstrated that antigenic profiles revealed differences between old and juvenile regions of *S. lacrymans* indicating that these may represent 2 different morphological types of mycelium. Recently however, Schmidt and Moreth-Kerbernik, (2000) designed species-specific oligonucleotide polymerase chain reaction (PCR) primers for *S. lacrymans* and *S. himantiodes* and found that in both field and laboratory samples each primer selectively detected its target organism. No cross-reaction occurred with other decay fungi, therefore establishing that species-specific priming PCR could readily identify *S. lacrymans*.

1.2.6. Dry rot treatment

The first priority when dealing with a dry rot infestation is to identify and eliminate the source of dampness and to provide good ventilation. *S. lacrymans* mycelia stop growing when exposed to draughts which then reduce the relative humidity of the atmosphere (Eaton and Hale, 1993). Brown *et al.* (1968) found that *Serpula* is capable of growing into wood blocks and causing weight loss at 90% relative humidity but not at 82%. The mycelium will grow into non-woody materials as well as spreading into wooden flooring, doorframes, skirting boards and staircases. The ability of *Serpula* to penetrate brickwork and plaster may result in infection of joists and other structural timbers in adjacent rooms and buildings. The masonry must be sterilised in addition to eradicating the infestation in the timber. All decayed timber up to 0.45 meters beyond the last point of infection must be removed and burnt. The surfaces of all brickwork and plaster must be cleaned and then heat-treated to help dry the material and kill any sub-surface mycelium. A fungicidal solution must then be applied to all surfaces.

Water-soluble preservatives are suitable for the treatment of absorbent surfaces. Timbers that remain *in situ* must be treated with a preservative and all replacement timber must be pressure impregnated with water or solvent based preservative prior to installation (Eaton and Hale, 1993). The traditional eradication strategy involves removal of timber and plaster and use of considerable amounts of chemicals to treat remaining and replacement timbers. Treated wood, however, is very expensive and the eradication process involves a lot of inconvenience for the inhabitants while the work is carried out.

1.2.7. Problems with chemicals

Chemicals used to inhibit, or destroy wood decay fungi have certain characteristics which may make them seem environmentally objectionable. A criticism of any chemical preservative is that it is biocidal and therefore potentially dangerous to humans other animals and plants (Eaton and Hale, 1993). Three types of preservative chemicals, the arsenicals, creosote and products based on pentachlorophenol (PCP) have been scrutinized most intensely in recent years. Approval to use the widely used tri n-butyline oxide has been withdrawn from the UK (Carey, 1992). An effort to reduce usage of the dioxin hexachlorodibenzo-p-dioxin (which is believed to be teratogenic in rats) is desirable as well as reducing the hazards associated with arsenic and chromium in CCA preservatives, which are more acute prior to impregnation.

1.2.8. Alternatives to chemicals

Elliot and Watkinson (1989) investigated the effect of an amino acid analogue, α-amino-isobutyric acid (AIB) on *S. lacrymans*. Impregnation of AIB into wood blocks inhibited decay by *Serpula* and AIB was found to inhibit hyphal tip extension and to

interfere with normal morphogenesis by increasing the frequency of mycelial branching. Since AIB could be translocated it had the potential to control dry rot in buildings. A large-scale fungal cellar test (Dobson *et al.*, 1993) demonstrated an inhibition of spread of *S. lacrymans* over wooden planks by AIB at concentrations of 1%. Some growth however, was still reported even at 10% (w/v) concentration.

In many cases one of the first indications that an infestation has occurred is the recognition of a musty odour in the building and development of a mushroom smell near to the site of decay. Indeed the release of volatile chemicals has been investigated as a means of detecting early stages of fungal decay. Bjurman and Kristensson (1992) used compounds such as ethyl benzene, furfural, acetone, ethanol and 1-methyl-2-propanol as a volatile fingerprint to aid identification. However, the problem with differential expression of volatiles caused by substrate competing fungi or environmental conditions is likely to be an obstacle. Similarly, snifferdogs have been trained to detect dry rot and can find 90-100% of cases prior to any damage occurring. In Sweden, dogs have been used to detect decay in poles and moulds in buildings (Koch, 1991).

The sensitivity of *S. lacrymans* to heat has also been exploited as a heat treatment method. Koch *et al.* (1989) and Koch (1991) developed a heat treatment strategy that could be combined with other general improvements in building maintenance. Affected parts of the building are heated at 40°C for 24 hours with the aim of killing the mycelium in the timber and masonry. Insulation material is placed along the façade of scaffolding erected outside the house. Temperature sensors are placed at appropriate points especially in places difficult to heat, for example in the centre of brick walls.

Warm air from an oil or gas burner is led into the insulated area both indoors and on the outside of the façade under the insulation. The use of chemicals is significantly reduced and the time spent on treatment is only a few days and the cost is between 10-50% lower than traditional methods of treatment dependent on extent and situation of attack.

Clearly new approaches to chemical preservation are required. The development and application of wood biotechnological techniques along with financial and environmental concerns over chemical preservation treatments has led to serious interest in the use of organisms to control wood decay fungi, including *S. lacrymans* (Freitag *et al.*, 1991).

1.3. Biological control

Baker and Cook (1974) defined biological control as:

"the reduction of inoculum density or disease producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host or antagonist or by mass introduction of one or more antagonist."

Freitag *et al.* (1991) introduced a more specific term for biological control involving a wood product as 'biological protection'. They felt that the control agent could be introduced into the wood prior to colonisation by the decay agent, for example, by inoculation into a hole drilled in the wood, so protection would be more appropriate than having to control established decay.

The concept of using biological agents to protect crops and animals is not a new one. Selection of resistant strains of crops is possibly the oldest practical application of

biological control and this practice could possibly date back as early as 8-10 000 BC (Coppel and Mertins, 1977). Similarly, domestication of wild felines and their usage as biological control agents by the ancient Egyptians to suppress mice and rats in their granaries is another early practical application. Chinese citrus growers utilised the red ant as a protective agent on their fruit trees and Groff and Howard (1924) noted that the large aggressive ants built their nests within the trees and drove off other insects that invaded their territory. By running bamboo pathways between the trees whole orchards were protected in this manner. The utilization of biological control in agriculture as a major means of crop protection became a reality in the early 1980s. American citrus growers in California scientifically evaluated the possibility of crop protection from scale insects, mealybugs and other problem pest infestations by the introduction of natural enemies of these pests. This resulted in the importation of specific predators to decrease pests in environments to which they are not native and perhaps the most successful illustration of this concerns the cottony-cushion scale insect, *Icerya purchansi*, which is a pest of citrus trees in California (De Bach, 1974). A search for natural enemies was carried out in Australia and New Zealand, the origin of the plant crop, and it was found that a parasitic fly *Cryptochaetum iceryae* and a predatory ladybird *Vedalia cardinalis* were natural enemies which could be subsequently established in California. The result of importation of *Vedalia* beetle was total elimination of scale as a major problem within 2 years. From the 1980s biological control has been used in increasing agricultural systems with varying degrees of success and more recently in the area of post harvest preservation.

1.3.1 Biological control as a means of protecting wood products

A number of soil fungi have been suggested as possible biocontrol agents for countering the root and butt rot of conifers caused by *Heterobasidium annosum*. Hyppel (1968)

screened 85 isolates of basidiomycete soil fungi against *H. annosum* and found that 40% of the isolates inhibited *H. annusom*. Fungicidal effects however were not generally seen. *T. viride* was also found to overgrow *H. annosum* on agar, soil and wood substrata in the laboratory (Rishbeth, 1950). The successful biological control method developed, however, employed the weak pathogen *Peniophora gigantea* (Rishbeth, 1963) which was able to become established in freshly cut stumps and in doing so excludes *H. annosum*. Encouraging results have emerged with some species of *Trichoderma* being used as protectants of pruning wounds especially for hardwood timber species. *T. harzianum* has been shown to exclude wood invading basidiomycetes in wounds on red maple (Pottle *et al.*, 1977). Grosclaude *et al.* (1973) found that by applying *T. viride* spores immediately after pruning, plum trees were completely protected against silver leaf disease (*Chondrostereum purpureum*). In field tests *T. viride* significantly decreased invasion by decay fungi *Coniophora puteana* and *C. purpureum* when inoculated into wounds on trunks of beech (Mercer and Kirk, 1984).

The possibility of protecting freshly felled timber against decay has been reported as far back as 1948 (Scheffer, 1969). Lindgren and Harvey (1952) found good establishment of *T. viride* on Southern pine logs especially when the timber had previously been treated with a low concentration of a fluoride containing preservative. Shields and Atwell, (1963) found that inoculating *T. viride* onto the end-grains of logs of white birch completely prevented the progress of artificially introduced decay fungus, *Polyporus adustus*. When applied as a pretreatment, *T. viride* was found only to be effective at excluding *P. adustus*. However, the *T. viride* could be applied up to three months after inoculation of the much slower growing basidiomycete *P. hirsutus* and still be effective in preventing colonisation (Hulme and Shields, 1975). In a survey of

common saprophytic fungi found to be capable of preventing primary colonization by species of *Armillaria*, *Trichoderma* emerged as the most antagonistic towards *Armillaria* on freshly cut branches of *Pinus radiata* and has been used commercially (Li and Hood, 1992). Decay of unsterile logs of Balsam fir could be significantly reduced by application of an aqueous suspension of mycelial fragments of *Cryptosporiopsis* sp. (Stillwell, 1966). Stillwell *et al.* (1969) isolated and characterised a broad-spectrum antibiotic from an isolate of *Cryptosporiopsis*. They also established its *in vitro* activity to a range of fungi, including fungi that decay wood (Stillwell and Hodgson, 1968). Lapetite (1970) used several bacterial isolates as effective pretreatments when protecting beech wood against white rot fungi. Preston *et al.* (1982) reported that six species of *Bacillus* were found to inhibit a number of white, brown and soft rot fungi in laboratory tests. Mode of action for most of the isolates was apparently through secretion of diffusible metabolites, which inhibited the fungi at a distance.

N. lepideus is the most common basidiomycete causing internal decay of creosoted pine poles in Britain (Morris, 1983). In North America, *Poria carbonica* causes a similar problem in creosoted Douglas fir poles (Giron and Morrell, 1989). Ricard and Bollen, (1968) used *Scytalidium* spp. to inhibit *P. carbonica* in Douglas fir poles and reported that an antibiotic produced by *Scytalidium* was responsible for control of the wood decay fungi. Without exception, the biological control agents employed in-service pole studies have been of the genera *Trichoderma* and *Scytalidium*, in most cases used in combination.

A Swedish bioprotection formulation, Binab T, was developed by Dr. J. Ricard and has been marketed in Europe for protecting Scots pine and later commercialised for use in the United States. The pellets of Binab contain mixtures of the mycelium and spores of

naturally occurring species of 3 antagonistic fungi, *T. harziunum* 25, *T. polysporum* 206039 and a *Scytalidium* spp. The potential use of Binab pellets include suppression of wood infesting organisms such as *Poria carbonica* and *N. lepideus* for utility poles, fence posts and wood playground equipment. Tests on Southern pine and Douglas fir suggest that this bioprotectant cannot completely control the numerous decay fungi associated with these species (Morrell and Sexton, 1988). In addition the agent was unable to completely eliminate decay fungi already established in the wood, nor did it perform well against white rot fungi (Zabel *et al.*, 1982; Graham and Corden, 1980; Eslyn, 1970). Bruce and King (1983) found that wood blocks could be protected from *N. lepideus* by *Trichoderma* sp. even after killing the *Trichoderma* and leaching the wood. Isolations from the infected regions of poles demonstrated that while *Trichoderma* was able to establish itself in the test poles, its distribution was influenced by the occurrence of other non-decay fungi and even though it appeared to affect the development of *N. lepideus,* areas were left uncolonised by the biological control agent (Bruce and King 1986 a,b). However, Bruce *et al.* (1991) have demonstrated that wood removed from poles 7 years after initial inoculation with Binab FYT pellets were still able to resist attack by decay fungi.

1.3.2 Biological control; limitations of use in wood

There is a body of opinion that control of basidiomycetes by organisms such as *Trichoderma* is, at best, transient and the usefulness of biological control may lie in short-term protective measures rather than longer term treatment (Morris *et al.*, 1992; Schoeman *et al.*, 1999). Biological control of decay in wooden structures has been limited by the fact that a wide range of organisms that are influenced by either the wood type or the environmental conditions can attack wood. Wood, depending on its intended use, may require to be protected for varying lengths of time ranging from a

few months to many years. As wood is often used as structural material any treatment system must not compromise the strength of the timber, so the range of potential control agents is naturally limited to those organisms which will not attack the structural polymers of the wood. The method and timing of application is also often a critical determinant of the success of any biological control system and therefore development of suitable delivery systems provides an additional challenge to the biological control of wooden structures (Bruce, 1998).

1.4. *Trichoderma* species

Among fungal antagonists that have been researched in the area of biological control *Trichoderma* spp. have proven to be the most successful. *Trichoderma* are soil fungi that occur ubiquitously and they comprise a group of fast-growing Hyphomycetes that are extremely common in agriculture, prairie, forest, salt marsh and desert soils in all climatic zones (Danielson and Davey, 1973; Domsch *et al.*, 1980). They are particularly prevalent in the litter of humid, mixed hardwood forests, comprising a minor component of the microbiota in the initial colonization but subsequently becoming more dominant (Papavizas, 1985). The widespread occurrence and effective potential of *Trichoderma* species can be associated with several factors including their metabolic versatility, resistance to microbial inhibitors and their antagonism to other microbes. The dominance of *Trichoderma* species in soil following fumigation is well known and is probably due to their inherent resistance to fumigants and their enhanced ability to colonise in the absence of competitive microorganisms. This remarkable broad-based tolerance of *Trichoderma* to growth inhibitors of both microbial and abiotic origin presumably facilitates their effective colonization of soil (Papavizas, 1985).

1.4.1 *Trichoderma* in agriculture

Trichoderma has been studied extensively as potential control agents for a wide range of plant pathogens in agricultural systems. The potential use of *Trichoderma* as biocontrol agents of plant pathogens was first suggested more than 50 years ago by Weindling (1932) who demonstrated the parasitic activity of members of this fungus to pathogens such as *Rhizoctonia solani*. Wells *et al.* (1972) were the first to report field control of *Sclerotium rolfsii* by infesting soil with *T. harzianum* grown on an autoclaved mixture of ryegrass seeds and soil. Grosclaude *et al.* (1973) demonstrated the effectiveness of *T. viride* against *Chondrostereum purpureum*, the cause of silver leaf disease of fruit trees by applying conidia of *T. viride* to wounds on shrubs and trees during cutting by means of special pruning shears. Tronsmo and Dennis (1977) were able to protect strawberry plants in the field against storage rot by spraying strawberry plants with aqueous suspensions of conidia of *T. viride* and *T. polysporum*. Although biocontrol was effective as sprays along with a fungicide, the level of control was not adequate from a commercial standpoint. Good biocontrol of damping-off caused by *Rhizoctonia solani* and *Pythium* spp. was achieved by treating seeds of peas and radishes with conidia of *T. hamatum* (Harmen *et al.*, 1980). Improved stands and yields were also obtained in *R. solani*-infested fields by planting soyabean seed treated with *T. pseudokoningii* (Wu, 1982) and seed of corn and soyabean treated with *T. harzianum* (Kommedahl *et al.*, 1981).

The last 20 years has seen a move from laboratory and field test to commercial products, as products that utilize Trichoderma as the active biocontrol agent enter the market. *T. viride* and *T. polysporum* have been incorporated into products sold in France and the United Kingdom for the control of silver leaf disease on trees and *Verticilium* wilt in mushrooms (Ricard, 1981). A *T. virens*-based agent marketed as

Gliogard™ was the first biocontrol agent to be awarded US Environmental Protection Agency approval. It is recommended for the control of *Rhizoctonia solani* and *Pythium ultimum*, the causal agent of damping off of ornamental shrubs (Lumsden and Locke, 1989) and the Southern blight of carrot and tomato caused by *Sclerotium rolfsii* (Ristaino *et al.*, 1994). *T. harzianum* preparations have also more recently entered the market as agents for the control of other plant diseases. A product based on *T. harzianum* strain T39 and sold under the brand name Trichodex™ 25P is marketed for the control of grey mould (*Botrytis cinerea*) on grapevines and sundry vegetables in several countries (Kubicek and Harmen, 1998). Integrated control application of fungicides with the biocontrol agent achieved as effective suppression of the growth of grey mould as that obtained by the fungicide alone (Kubicek and Harmen, 1998).

1.4.2. Biological control of *S. lacrymans* by *Trichoderma*

Score and Palfreyman (1994) screened a number of *Trichoderma* isolates for their ability to kill *S. lacrymans* in cross plate interactions using a standard medium and minimal essential medium to mimic the carbon : nitrogen ratio in wood. *T. harzianum* 25 proved most efficient at killing *Serpula*. Altering the levels of nitrogen and iron indicated that these two elements could prove important in the biological control since *Serpula* may utilize iron as part of a non-enzymatic complex used to initiate wood decay. This is important because *Trichoderma* release compounds known as siderophores (Srinivasan, 1993) that sequester iron and if the iron can be removed from wood by such a mechanism, it will be unavailable for the decay process of *S. lacrymans*. Due to the problems of extrapolating data from enriched media interactions to real life situations Score *et al.* (1998) screened 30 *Trichoderma* isolates for their ability to inhibit and ultimately kill 10 isolates of *S. lacrymans* in standard dual culture

plates and medium containing Pine sapwood sawdust. The results demonstrated that 26 of the *Trichoderma* isolates were antagonistic towards *Serpula*. The potential use of *Trichoderma* species as bioprotectants or biocontrol agents was investigated using a small wood block system utilizing pine sapwood stakes. The results indicated that the presence of *Trichoderma* (viable or non-viable) prevented the colonization of the blocks by *S. lacrymans*. However, the *Trichoderma* species did not stop the decay of blocks initially colonized by *Serpula* and the stake system showed that although *Trichoderma* colonisation of the stakes could prevent *S. lacrymans* decay it did not stop the spread of *Serpula*.

Doi and Togashi (1990) found strands/mycelium of *S. lacrymans* growing into the soil under the floor of dry rotted houses in Japan. They reported that it was more efficient for *S. lacrymans* to germinate on the soil surface than on the wood surface as the fungus could metabolise the nitrogeneous substances and carbon materials in/on soil and subsequently the growing mycelium attacked the wood in the houses. Based on these observations Doi and Yamada (1992) suggested treating the soil as well as the timber with preservative to prevent dry rot damage of buildings in Japan. Therefore, they investigated the utilisation of *Trichoderma* spp. instead of preservatives for soil treatment. In dual culture studies all strains of *Trichoderma* tested were antagonistic or aggressively dominant to *S. lacrymans* and inoculation of soil samples with specific *Trichoderma* species could prevent or reduce the weight loss of *S. lacrymans* infected wood blocks placed on the soil. Doi and Yamada (1992) felt that their work indicated *Trichoderma* spp. have potential as natural agents for biological control of growth of *S. lacrymans* in soil.

1.5. *Trichoderma* mechanisms of action

Through years of extensive investigation it has been determined that *Trichoderma* species antagonise other fungi through a variety of active mechanisms. *Trichoderma* spp. also employ a variety of passive mechanisms, such as an ability to dominate substrates through fast growth rate, prolific spore production, metabolic versatility and tolerance of environmental stresses especially chemicals (Eveleigh, 1984).

1.5.1. Competition for nutrients

It was suggested that *Trichoderma* spp. inhibit the attack of wood destroying fungi by removing non-structural carbohydrates from the wood, which are necessary for rapid colonization and initiation of decay by wood destroying fungi (Hulme and Shields, 1970). Thereby the *Trichoderma* spp. occupy the ecological niche of the target organisms so preventing their establishment. Depleting or exhausting readily assumable nutrients such as simple sugars can create an environment unsuitable for the germination of competitors' spores.

1.5.2. Mycoparasitism

Mycoparasitism is a multistage behavioural process in which *Trichoderma* spp. are chemotropically attracted to the decay fungus, on contact they coil their mycelium around or grow along the host's cell wall and excrete lytic enzymes, such as laminarinase and chitinase, which enable them to degrade the host's cell wall and utilise its nutrients (Haran *et al.*, 1996)

1.5.3. Soluble metabolites

Many people have reported the effect of soluble metabolite production by *Trichoderma* spp. against target fungi (Taylor, 1986). The effect of these inhibitors can be clearly

seen in dual cultures. Typically, a zone of inhibition where growth has slowed or ceased becomes apparent in the colony of the target fungus as the metabolites secreted by the antagonistic fungus diffuse out into the agar. Dennis and Webster (1971a) found that the ability to produce non-volatile metabolites varied between isolates within the same species aggregate of *Trichoderma*, as well as between isolates of different species. They also found that the effect of these metabolites on target fungi varied considerably with *H. annosum* the most susceptible *and Fusarium oxysporum* the most resistant.

1.5.4. Siderophores

Trichoderma isolates can produce both phenolate and hydroxamate siderophores that allow effective capture of iron from the immediate environment (Srinivasan 1993). It is possible that *Trichoderma* inhibit other fungi by actively sequestering iron in this way and depriving basidiomycetes of valuable iron and ultimately reducing their wood decay capacity.

1.6. *Trichoderma* VOCs

Studies in a controlled environment can help to estimate the potential of an organism and Martin (1963) (cited in Hutchinson, 1971) tested 63 fungi for volatile influence on 5 assay fungi. Cultures of the 63 fungi were grown on malt agar in Petri dishes until the surface of the agar was covered. The lid was then replaced with a sheet of cellophane and inverted over another dish of malt agar that had been inoculated with one of the 5 assay fungi. Two of the 63 fungal species were *Trichoderma*, *T. viride* and *T. koningii*. The results demonstrated that *T. koningii* had no effect on the growth of any of the five assay fungi tested. *T. viride* however strongly inhibited 2 of the fungi, *B. cinerea* and *Chaetomium globosum*. Martin (1963) concluded that volatile compounds associated with metabolic pathways such as ethanol, ethyacetate, acetaldehyde or carbon dioxide

may be responsible for the biological effect caused by the volatiles from the test fungi although this could not be guaranteed.

Dennis and Webster (1971b) reported that isolates of *Trichoderma*, including isolates from the same species group, produced a range of volatile metabolites that had different effects on a range of test fungi. The active isolates were all characterised by a definite coconut smell referred to by Bisby (1939) and Rifai (1969). However, there were 2 isolates, which possessed the smell but did not bring about significant inhibition of growth. The active metabolites caused stunted mycelial growth in all susceptible test fungi, but there was no correlation between sporulation and volatile production. Dick and Hutchinson (1966) reported volatile metabolites produced by *Trichoderma* isolates to have fungistatic effects, although Bilai (1956) earlier reported fungicidal volatiles by some *Trichoderma* isolates. Bilai (1956) observed an anti-bacterial effect of volatile substances of various *Trichoderma* cultures which were more active during 2 phases of the *Trichoderma* growth, namely at 5-10 days and 15-20 days.

Dennis and Webster (1971b) tentatively identified acetaldehyde as one inhibitory metabolite of *T. viride*. They found that 100 parts per million (ppm) of acetaldehyde caused slight inhibition of *F. annosus* but 500 ppm of acetaldehyde was required to cause complete inhibition of the target fungus. Robinson and Garrett (1969) reported that 15 ppm acetaldehyde caused appreciable retardation of spore germination of *C. elegans*. Dennis and Webster (1971b) concluded that *Trichoderma* isolates probably produced more than one active volatile or produced the same complex of metabolites in different proportions to bring about their inhibitory action.

Ethanol and carbon dioxide, which are common respiratory metabolites and breakdown products, have also been increasingly reported as producing remarkable effects on fungi at low concentrations. Hutchinson and Cowan, (1972) found that gases from a strain of *T. harzianum* inhibited the growth and sporulation of *A. niger* and *P. rhododendri*, which were responsible for the inhibition of coloration and rate of elongation of lettuce seedlings. Analysis of the headspace gases of *T. harzianum* found that carbon dioxide and ethanol were the only metabolites recorded in more than trivial amounts. Hutchinson and Cowan (1972) felt that the effects seen could be attributed to either amounts of ethanol or carbon dioxide formed by *T. harzianum*. Taminimi and Hutchinson (1975) reported that gaseous metabolite inhibition of a range of assay fungi by volatile organic compounds (VOCs) from different species and strains of *Trichoderma* were explained by differences in the rate of production of carbon dioxide under the conditions with acetaldehyde or ethanol also contributing to the inhibitory effect. However, they did not discount the possibility of metabolites that were not as yet identified contributing to the effects seen.

Most reported interaction studies have been carried out at 25°C, which is higher than the ambient temperature in external environments temperate climates. *Trichoderma* spp. are capable at growing over a large range of temperatures, but no-one until Tronsmo and Dennis in 1978 had investigated how they would grow at much lower temperatures. Tronsmo and Dennis (1978) tested the effect of temperature on effectiveness of *Trichoderma* spp. at suppressing *B. cinerea* and *Mucor mucedo*. The production of inhibitory volatile metabolites was tested at 5, 10 and 20°C, and the concentrations of oxygen and CO_2 were also determined. The CO_2 was removed from the system by placing 2 circular discs containing sodium hydroxide (NaOH) to adsorb

the CO_2. The results demonstrated that as the temperature increased so did the number of *Trichoderma* species producing inhibitory volatile compounds which caused stunted mycelial growth and frequent branching in both *B. cinerea* and *M. mucedo*. Addition of NaOH showed there was slight decrease in inhibition but never more than 5% which proved that most of the inhibition was caused by volatiles other than CO_2 as reported by Taminimi and Hutchinson (1975). Tronsmo and Dennis (1978) felt it was unlikely that a single component was responsible for the inhibition seen as the test fungi showed different sensitivities to compounds produced by the strains of *T. viride*.

The main constituents of the coconut aroma previously linked to inhibitory strains of *Trichoderma* (Bisby, 1939; Rifai, 1969; Dennis and Webster, 1971b) were identified as 6-pentyl-pyrone and the closely related analogue 6-pentenyl-pyrone (Collins and Halim, 1972; Moss *et al.* 1975). Claydon *et al.* (1987) found that the pentyl analogue, the major product of 6-pentyl-pyrone and 6-pentenyl-pyrone, had inhibitory properties to a range of fungi and considerably reduced the rate of damping off in lettuce seedlings by *R. solani in vitro*. They concluded that the main mode of action of the pentyl analogue was fungistatis, apart from *R. solani* and *R. cerealis* where they felt the alkyl pyrones acted as paramorphogens, changing the spatial distributions of the organisms biomass but not its rate of production.

Bruce *et al.* (1984) reported that volatiles from FYT *Trichoderma* were solely responsible for a fungicidal effect on the wood decay fungus *L. lepideus*. Total inhibition of growth of *L. lepideus* was obtained followed by total disruption and cell wall lysis. Subcultures produced no re-growth of *L. lepideus* showing that the fungus was no longer viable. Prior to this cell lysis had only been seen when the antagonist and

target fungi were grown in the same agar plate and overgrowth of the target fungus by the antagonist resulted in mycelial lysis in the target organism.

1.6.1. Volatile identification

Bruce *et al.* (1996) examined the ability of *Trichoderma* spp. to produce VOCs over a four-week period of growth and the inhibitory effect of these volatiles against 4 basidiomycetes over the same period was also assessed. After trapping on tubes filled with chromatography packing material, VOCs were analysed on an integrated automated thermal desorption gas chromatograph - mass spectrometer (GC-MS). A total of 72 separately identified compounds were recovered although production of any single compound was time dependent. The inhibitory effect of the VOCs against the 4 basidiomycetes varied dependent on the age of the *Trichoderma* culture. Highest levels of inhibition were produced by cultures which were 1-2 weeks old at which time 85% inhibition of *L. lepideus* was recorded (Bruce *et al.*, 1996). During this time period there was also a major switch from alcohol to aldehyde and ketone production. *L. lepideus* has consistently been recorded to be more susceptible to *Trichoderma* volatiles than other wood decay fungi (Bruce *et al.*, 1984, 1987).

Wheatley *et al.* (1997) examined the production of VOCs by 2 *Trichoderma* isolates grown on sand infused with either malt extract broth or a minimal medium with the carbon : nitrogen levels similar to that found in Scots pine wood. The inhibitory effect of the VOCs from the 2 *Trichoderma* isolates was again tested against 4 common wood decay fungi. The VOCs were analysed by GC-MS and a total of 45 individual compounds were recorded. Principal component analysis was used to establish that the production of individual VOCs was dependent on both the *Trichoderma* isolate and the growth media type. Srinivasan *et al.* (1992) have also shown that inhibition of selected

basidiomycetes by VOCs was influenced by the media type used, the species of antagonistic *Trichoderma* and that some target fungi were more susceptible than others. Although VOCs from both *Trichoderma* spp. grown on a malt agar medium consistently inhibited the growth of all 4 target fungi, effects were negligible when isolates were grown on minimal agar medium. Statistical analysis implicated 5 compounds that were most likely to be involved in the inhibition of the wood decay fungi. They were 2-propanone, also known as acetone, 2-methyl-1-butanol, heptanal, octanal and decanal. Due to the fact that little inhibition was seen on the minimal agar medium it was concluded that it is most unlikely that volatile production by *Trichoderma* isolates *in situ* in wood would act as long-term protection strategy for biocontrol agents in wood. However, it is possible that the chemicals identified may be used at appropriate concentrations as fumigants in wooden structures (Wheatley *et al.*, 1997).

Humphris *et al.* (2001) tested four of the five compounds implicated in the inhibition of wood decay fungi by *Trichoderma* volatiles (Wheatley *et al.*, 1997) against four basidiomycetes. The results showed that the 2 aldehydes (heptanal and octanal) were effective in inhibiting all 4 wood decay fungi at concentrations as low as 25 $\mu l\ ml^{-1}$.

1.7. Aims

Volatile compounds undoubtedly play a significant role as one of many environmental factors affecting fungal development and may determine the distribution, rate of growth and mode of development of these organisms in nature. All living organisms produce volatile metabolites that can have a significant effect on the ecological balance within their community. Whether these compounds are stimulatory or inhibitory depends on their concentration and the specific sensitivity of the responding fungus (Bruce *et al.*, 1996; Wheatley *et al.*, 1997; Mackie and Wheatley, 1999).

It is intriguing that compounds as simple as aldehydes and alcohols may reduce fungal growth and that even at very low *in situ* concentrations may produce effects on the ecological balance of ecosystems. If they are indeed major determinants in inhibiting fungal growth then a number of questions need to be investigated. For example, do these organic compounds interfere with fungal metabolism by blocking effects of some critical enzyme, thereby disrupting growth and/or mode of fungal differentiation? Alternatively, do they control growth at the more fundamental level of gene regulation?

The major aim of the studies in this PhD will be to evaluate the production of volatile organic compounds by *Trichoderma* species and assess their importance as a mechanism of control of *S. lacrymans*.

This will be achieved by initial screening tests to determine whether *Trichoderma* spp. produce volatile effects against *S. lacrymans* and if produced whether such interactions are specific to certain *Trichoderma* isolates or target strains of *S. lacrymans*. Srinivasan *et al.* (1992) & Score (1998) have both shown the importance of the nutrient composition on the effectiveness of biological control by *Trichoderma* isolates. If

VOCs from *Trichoderma* are to make a significant contribution in any practical biological control application against *S. lacrymans* the inhibitory volatiles will need to be produced within media that resemble as closely as possible the nutritional status of selected wood species.

The first objective of this study will therefore be to develop a suitable screening system on sawdust and a variety of synthetic media to determine which of a range of *Trichoderma* isolates produce an inhibitory VOC effect on a selection of *Serpula lacrymans* isolates and to evaluate their relative sensitivities to this volatile stress.

While inhibitory volatiles produced by *Trichoderma* have previously been identified (Bruce *et al.*, 1996; Wheatley *et al.*, 1997), no volatile compounds have ever been identified from *Trichoderma* growing on wood based substrates. The growth substrate will undoubtedly have an effect on the range and/or quantity of volatiles produced by *Trichoderma* isolates (Srinivasan *et al.*, 1992; Score, 1998). Any volatiles identified from *Trichoderma* grown on malt extract media may not necessarily therefore also be produced when the *Trichoderma* is growing in wood. Identification of inhibitory compounds produced by the *Trichoderma* on sawdust or agar designed to better illustrate the nutritional status of wood may have significance in the biological control of *S. lacrymans in situ* within timber.

The second objective of the study will therefore be to identify those individual VOCs produced by the *Trichoderma* isolates that are most likely to be responsible for the inhibition of the dry rot fungus in wood.

Further studies will be required to evaluate the specific mode of action of *Trichoderma* volatiles against *S. lacrymans*. Successful biological control of dry rot may not necessarily require a system that kills the fungus but one that merely limits the wood decaying abilities of the fungus. To date no studies have ever been undertaken to establish how toxic fungal volatiles influence target species. Molecular analysis of *S. lacrymans* will be carried out to assess the target action of *Trichoderma* volatiles. While molecular studies have been used as tool for identification of *S. lacrymans* (Schmidt and Moreth-Kebernik, 1989b; Palfreyman *et al.*, 1991; Vigrow, 1992), no molecular studies have examined effects of biotic stress (including fungal VOCs) on *Serpula*.

The final objective of the study will therefore be to use suitable molecular biology methods to establish the mode of action of *Trichoderma* volatiles against *S. lacrymans* by assessing the effect of volatiles both on extracellular enzyme activity and protein production in the dry rot fungus.

CHAPTER 2: The effect of *Trichoderma* VOCs on the growth and extracellular enzyme activity of *S. lacrymans*

2.1. Introduction

Trichoderma species antagonise other fungi through a variety of active mechanisms, including: competition for nutrients (Hulme and Shields, 1970); mycoparasitism (Haran *et al.*, 1996); soluble metabolites (Dennis and Webster, 1971a; Taylor, 1986); siderophore production (Srinivasan, 1993) and volatile metabolites (Dennis and Webster, 1971b; Tronsmo and Dennis, 1978; Bruce *et al.*, 1996; Wheatley *et al.*, 1997). Such interactions are not necessarily inhibitory, can even result in stimulatory effects, and can be expressed in changes in growth rates and enzyme activity (Bruce *et al.*, 1996; 2000; Wheatley, *et al.*, 1997; Mackie & Wheatley, 1998). *Trichoderma* isolates have been reported to produce volatile organic compounds (VOCs), which have been proven to show fungistatic and/or fungicidal properties against brown and white rot fungi (Bruce *et al.*, 1984). Srinivasan *et al.* (1992) and Bruce *et al.* (1996) have shown that inhibition of wood decay fungi by *Trichoderma* VOCs is dependent on the type of growth media, the age of the *Trichoderma* colony and the species of target organism. The exact mode of antagonism *Trichoderma* employs to achieve this however, is not yet fully understood.

The effects of VOCs on fungal metabolism can be examined by monitoring the production of extracellular enzymes. Tyrosinase production in fungi is thought to be linked to pigmentation (Flurkley *et al.*, 1995; Jolivet *et al.*, 1998) where the enzyme catalyses two different reactions, firstly the hydroxylation of monophenols to o-diphenols and the oxidation of o-phenols to o-quinones, which in turn are polymerised to pigments (Espin *et al.*, 1999). Tyrosinase production in fungi is also linked to morphogenesis (Griffith *et al.*, 1994). Peroxidase enzymes have been implicated in the production of highly toxic compounds that are antifungal in nature and also in the formation of different mycelial formations and pigment production (Velazhahan and

Vidhyasekaran, 1994). White and Boddy (1992) found that phenol-oxidasing enzymes were correlated with the production of aerial mycelium in *P. radiata*. Although there are no reports of VOCs that inhibit these enzymes, Score *et al.* (1997) examined whether three enzymes (laccase, tyrosinase and peroxidase) were produced during basidiomycete confrontations with several deuteromycetes. This was assessed by performing a number of pairings between two brown rot fungi, *Serpula lacrymans* and *Coniophora puteana* with several deuteromycetes including, *Trichoderma* spp. and *Scytalidium* FY. The results indicated that there was a link between laccase and peroxidase activity and fungal antagonism with both these enzymes being detected in the interaction zones during the fungal pairings. Score *et al.* (1997) speculated that there was a connection between peroxidase release and mycelial invasion in *S. lacrymans* and that laccase activity in *C. puteana* was linked to pigmentation of the colony and formation of invasive mycelial fans.

The aim of this chapter was to use volatile mediated interactions to screen a range of *Trichoderma* isolates against a number of *S. lacrymans* isolates with the *Trichoderma* grown on a range of different media including sawdust. This will determine whether *Trichoderma* spp. produce volatile effects against *S. lacrymans* and if produced whether such interactions are specific to certain *Trichoderma* isolates or target strains of *S. lacrymans* and if such interactions influence the production of the extracellular enzymes cellulase, peroxidase and tyrosinase.

2.2. Materials and methods

2.2.1. Test fungi

Four *Serpula lacrymans* isolates were used in these experiments. These were chosen to identify if there was varying sensitivity between different strains. They were *Serpula lacrymans* (Schum. Ex. Fr.) S.F. Gray FPRL 12C, a standard laboratory culture, *Serpula lacrymans* (Schum. Ex. Fr.) S.F. Gray BF050, an Australian building isolate, *Serpula lacrymans* (Schum. Ex. Fr.) S.F. Gray H28 isolated from a decaying log in the Himalayas and *Serpula lacrymans* (Schum. Ex. Fr.) S.F. Gray Forfar, isolated from a building in Scotland. Stock cultures were maintained on 5% (w/v) malt extract (Oxoid, Hampshire, UK) / 2% (w/v) agar (Oxoid, Hampshire, UK) and stored at 4°C. Fresh culture material was established at approximately 3 month intervals from fridge stored stock material.

Nine *Trichoderma* stains were selected to screen for VOC activity; *T. aureoviride* (Rifai) IMI 91968, *T. harzianum* (Rifai) Scottish Institute for Wood Technology (SIWT) Culture collection 25, *T. harzianum* (Rifai) IMI 206040, *T. polysporum* (Link ex Pers.) IMI 206039, T. *pseudokoningii* (Rifai) SIWT 64, *T. koningii* (Rifai) IMI 54693, *T. viride* (Pers ex S.F. Gray) SIWT 70, *T. viride* (Pers ex S.F. Gray) SIWT 110 and a culture of *Trichoderma* (FY *Trichoderma*) isolated from Binab FYT pellets (Bioinnovation Ab Binab, Sigtuna, Sweden) containing *T. harzianum* IMI 204060, *T. polysporum* IMI 206039 and *Scytalidium FY* (Pesante) ATCC 16675. Stock cultures were maintained on 3% (w/v) malt extract agar (MEA) (Oxoid, Hampshire, UK) and stored at 4°C. Subcultures were routinely taken from these every three months for ongoing experiments.

2.2.2. VOC mediated interactions

A mycelial core (5mm in diameter) was removed from the periphery of a 1-2 week old plate of *Trichoderma* and centrally inoculated onto a plate of 3% MEA. Plates of 5% malt extract / 2% agar were similarly inoculated with a core of *S. lacrymans* (5mm in diameter). After removal of the lids, plates inoculated with the *S. lacrymans* were covered with a semi-permeable polyvinyl chloride cling film membrane (BDH, Dorset, UK) and placed on top of the plates inoculated with the *Trichoderma* isolates and sealed with Nescofilm™ (BDH, Dorset, UK). This experiment was also repeated but with the *Trichoderma* grown on a minimal media and Scots pine sawdust instead of malt extract (Fig.2.1), to determine whether production of VOCs was influenced by nutrient composition.

The minimal media had a carbon : nitrogen ratio similar to that typically found in Scots pine wood and was based on Hutterman and Volger (1973) consisting of 5g D-glucose; 1g potassium dihydrogen phosphate; 0.5g potassium chloride; 0.3g magnesium sulphate; 0.01g ferric sulphate; 0.008g manganese acetate tetrahydrate; 0.002g zinc nitrate hexahydrate; 0.05g calcium nitrate; 0.002g cupric sulphate; 0.008g ammonium nitrate NH_4NO_3; 0.013g L-asparagine (anhydrous) with 1.5% purified agar per litre. The L-asparagine was added to 1ml of sterile water and then added to the minimal media using a sterile syringe and filter after the media had been autoclaved and cooled to hand hot temperature.

Four g of autoclaved Scots pine sawdust (17% w/v moisture content) was wetted with 8ml of autoclaved ultra-pure water to bring the moisture content up to 120% (dry weight biomass), this left the sawdust waterlogged but with no free water in the Petri

dish (Appendix A, 1.1). The sawdust was then inoculated with the appropriate *Trichoderma* and incubated for one week before the plates containing cores of *S. lacrymans* were placed on top. This allowed the *Trichoderma* time to colonise the sawdust. In all experiments the *S. lacrymans* strains were grown only on MEA. These were exposed to the 9 *Trichoderma* isolates on 3 different media, MEA, minimal media and Scots pine sawdust, giving a total of 27 treatment combinations for each *Serpula* strain. Controls were also set up on three media types in an identical fashion except they contained no *Trichoderma* inocula on the bottom plate. All cultures were incubated at 21°C until the growth of the *S. lacrymans* on the control plates had nearly reached the edge of the plate (usually 7 days). Three replicates were set up for all tests and controls. Growth of the *S. lacrymans* was measured using a Digital Image Analysis System (Delta-T Devices, U.K.), and the area (cm^2) was recorded. Inhibition of growth of the *S. lacrymans* was recorded as the difference in mean growth area of *S. lacrymans* in the presence or absence of the *Trichoderma* cultures expressed as a percentage of the growth of the controls, using the calculation below.

$$\frac{\text{Mean area of control} - \text{Area of test}}{\text{Mean area of control}} \times \frac{100}{1} = \% \text{ Inhibition}$$

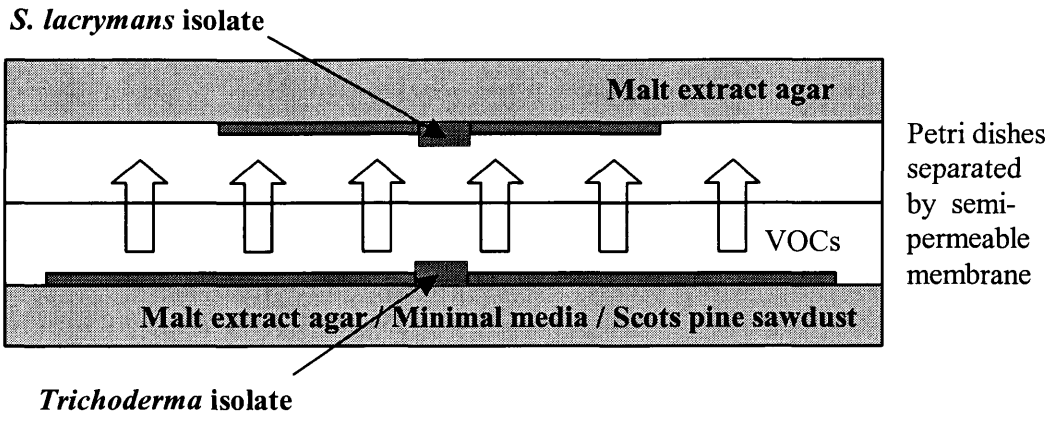


Figure 2.1. Experimental set-up for screening interactive VOCs.

2.2.3. One-way analysis of variance

A one-way analysis of variance (ANOVA) was carried out for each of the 27 test combinations, of each *S. lacrymans* strain. ANOVA describes the variation of the data when compared to the control cultures. The significance of the variance ratio was quantified by the *p*-value, showing whether or not there was significant inhibition of the test treatments compared with the controls.

2.2.4. Extracellular enzyme activity of *S. lacrymans* colony

The effects of the *Trichoderma* volatiles on the activity of 3 extracellular enzymes, tyrosinase, peroxidase and cellulase, was assessed in a manner similar as that described by White and Boddy (1992) and Thorn (1993). Identical tests were carried out as described in section 2.2.2 with the *Trichoderma* on 3% malt extract and the *S. lacrymans* on 5% malt extract agar. After the *S. lacrymans* cultures had been exposed to the *Trichoderma* isolates, the following enzyme tests were undertaken:

1. Tyrosinase - 5ml of 1.08% (w/v) p-cresol in ethanol was flooded onto the *S. lacrymans* culture and plates were left at room temperature for 24h to allow any colour reaction to develop. A red colour reaction indicated tyrosinase activity.

2. Peroxidase - 5ml of 1.26% (w/v) pyrogallol in distilled water, added to 0.4% hydrogen peroxide was similarly used to assess peroxidase production. The plates were left at room temperature for 24h to allow any colour reaction to develop. A brown colour reaction indicated peroxidase activity.

3. Cellulase – Sterile filter paper was placed on the bottom of the Petri dishes onto which 5% malt extract agar was then poured. Cellulose azure was sprinkled over the 5% malt extract and left to absorb into the media for 2 hours before the *S. lacrymans* core was inoculated. Plates inoculated with *Trichoderma* were inverted over the *S. lacrymans* plates. After the plates had been incubated in contact with the *Trichoderma* VOCs for 7 days, they were checked for release of cellulose azure dye into the media by looking at the colour on the filter papers. Release of a red coloration onto the filter paper indicated cellulase activity.

2.3. Results

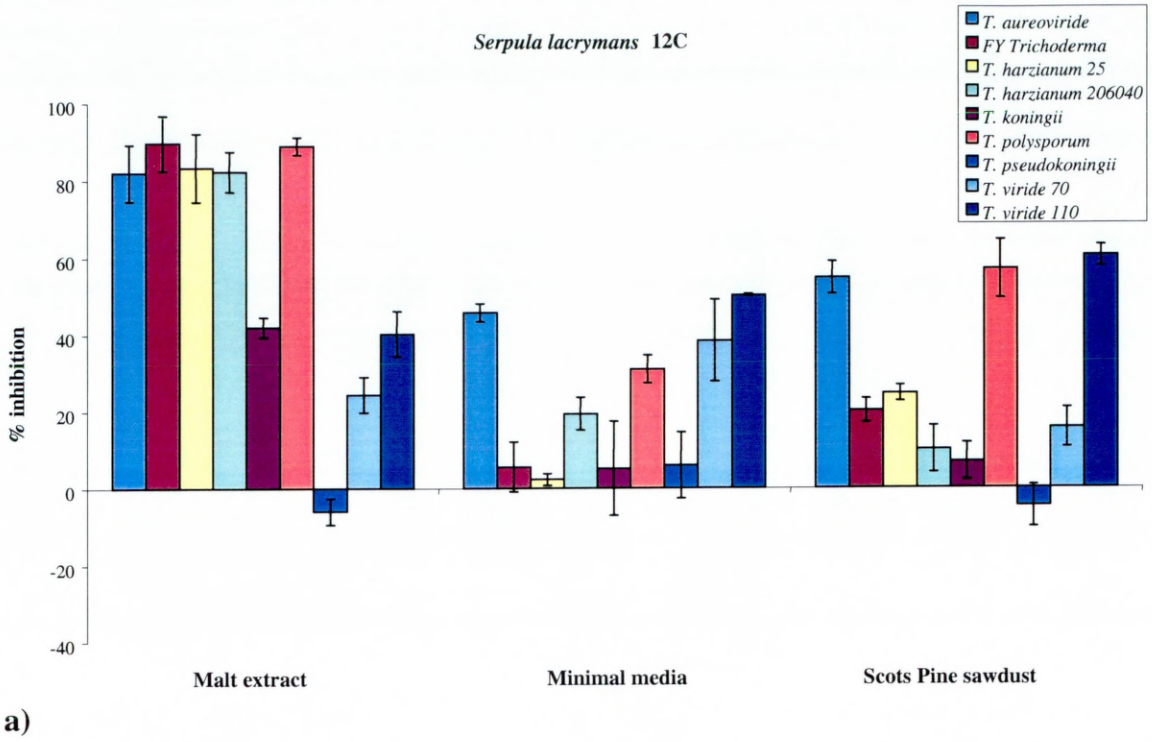
2.3.1. VOC mediated interactions

The levels of growth inhibition of the *S. lacrymans* isolates by VOCs from the nine *Trichoderma* isolates are shown in Figures 2.1a,b and 2.2a,b and the corresponding *p*-values from the statistical analysis are shown in Table 2.1. The results show that all except 10 of the 108 treatments (27 treatments for each *Serpula* strain) were inhibitory (Fig. 2.1a,b & 2.2a,b). The majority of the treatments were significantly inhibitory (Table 2.1) and of the 10 stimulatory treatments, only 4 were statistically significant at the $p \leq 0.05$ levels. They were *S. lacrymans* BF050 exposed to *T. pseudokoningii*, *T. viride* 70 and *T. koningii* grown on minimal media and *S. lacrymans* Forfar exposed to *T. viride* 70 on minimal media. The results also indicate that certain *Trichoderma* isolates are more potent producers of inhibitory VOCs than others. For example, *T. aureoviride* and *T. polysporum* were the only 2 isolates that produced significant inhibition of all *S. lacrymans isolates* on all media, whereas *T. pseudokoningii* produced the least inhibition of all isolates on all media, with inhibition never being above 15% and only 1 treatment (*S. lacrymans* BF050 exposed to *T. pseudokoningii* grown on malt) showing statistically significant inhibition. The mycelium of the *S. lacrymans* regularly produced a yellow pigmentation when inhibited about 50% or more by the *Trichoderma* VOCs.

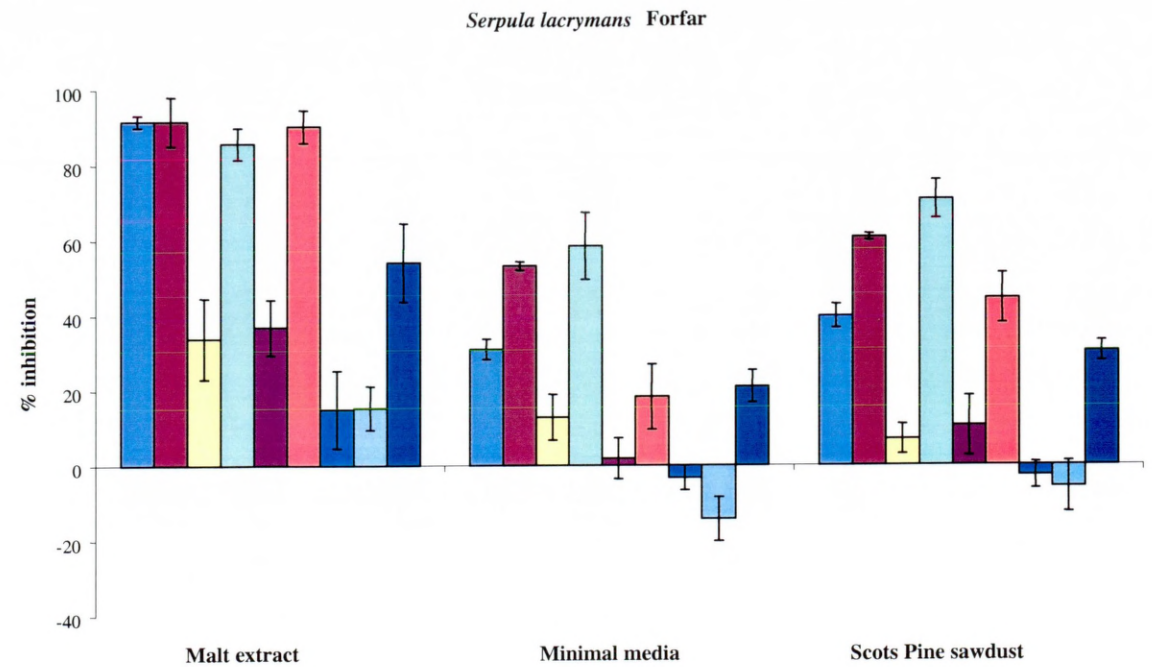
Volatile inhibition of all four *S. lacrymans* cultures was greater on the malt extract agar compared with the minimal media and the Scots pine sawdust. For example, *T. polysporum* VOCs inhibited the growth of *S. lacrymans* Forfar by 90% when grown on malt but only 18% on minimal media and 44% when on sawdust. An interesting anomaly was *T. viride* T110, which gave higher levels of inhibition when grown on sawdust and minimal media rather than malt for *S. lacrymans* H28 and 12C. Levels of

inhibition generally tended to be slightly greater when the *Trichoderma* cultures were grown on the sawdust media when compared with the minimal media, with the majority of growth stimulation being seen when the *Trichoderma* isolates were grown on minimal media.

There was also varying sensitivity between the *S. lacrymans* isolates, for example, *T. harzianum* 25 inhibited *S. lacrymans* H28 and 12C by 56% and 83% respectively but only inhibited Forfar and BF050 by 33% and 19% respectively. On the malt media H28 appeared to be the least susceptible to the *Trichoderma* VOCs with inhibition never rising above 78%, whereas Forfar and 12C appeared to be the most susceptible with inhibition up to 92% and 90% respectively. On the minimal media, the greatest percentage inhibition was *S. lacrymans* Forfar exposed to the VOCs of *T. harzianum* 206040. *S. lacrymans* H28 and BF050 seemed less susceptible with BF050 even being significantly stimulated by *T. koningii*, *T. pseudokoningii* and *T. viride* 70. On the sawdust, the sensitivities of the *S. lacrymans* isolates were more similar to one another than on the other 2 media.

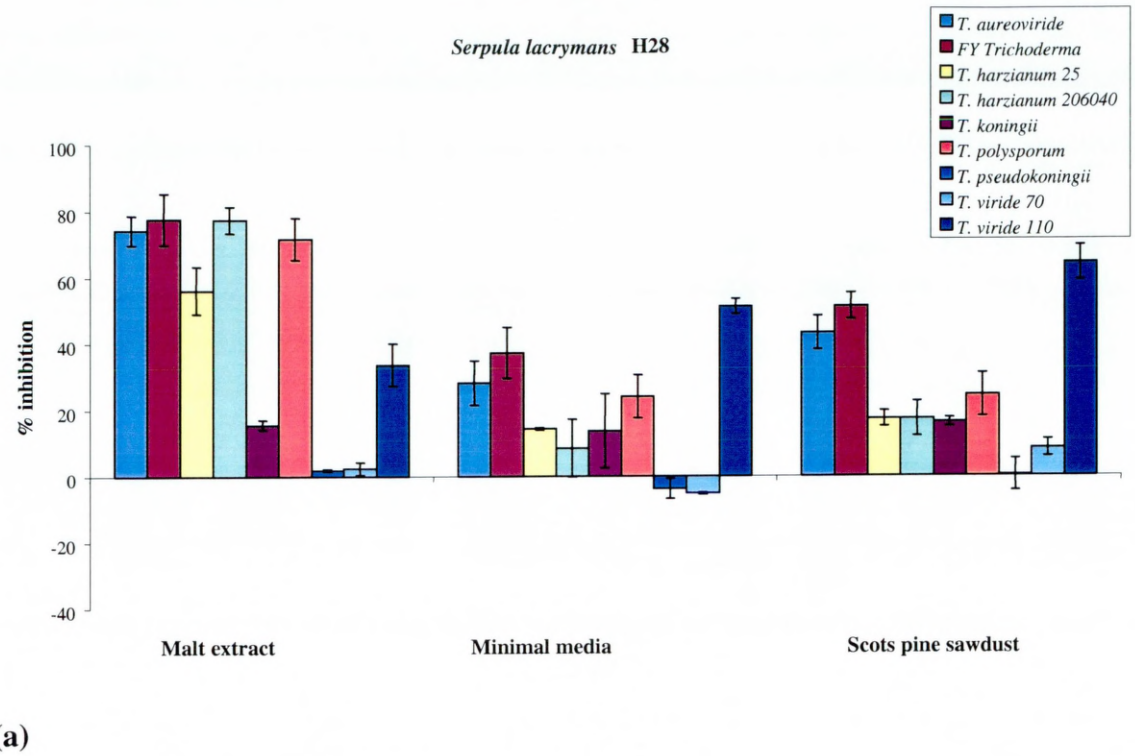


a)

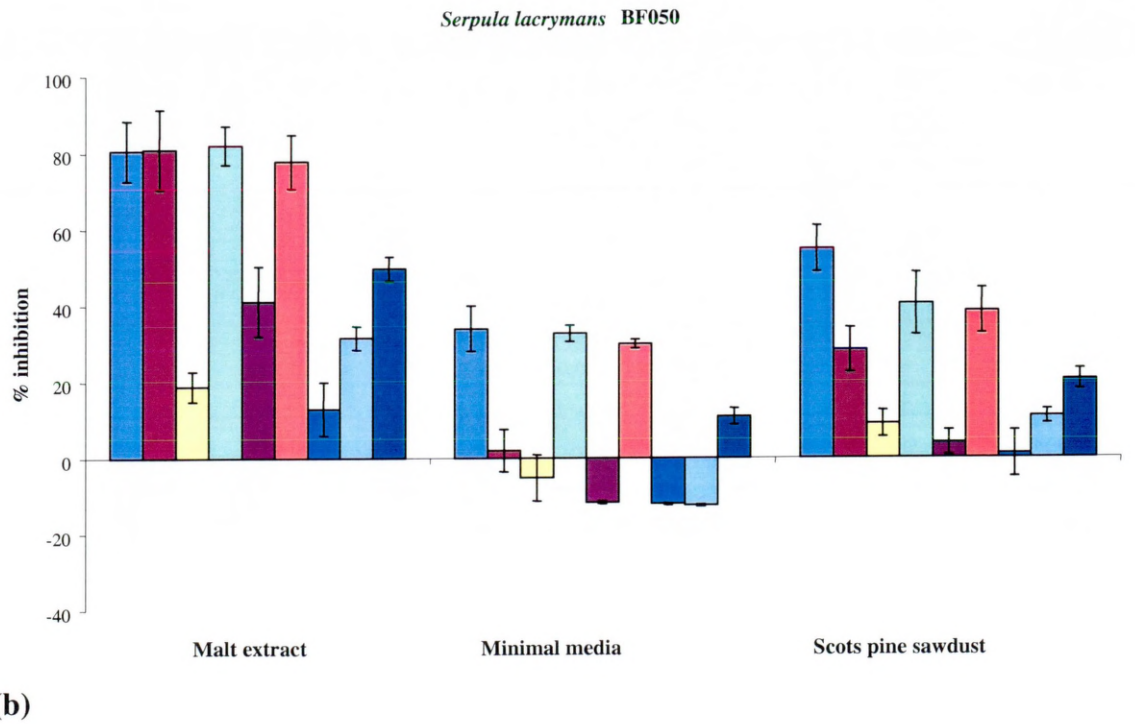


(b)

Figure 2.2. Growth inhibition as a % of the control, of *Serpula lacrymans* 12C (a) and Forfar (b) isolates exposed to the VOCs from nine *Trichoderma* isolates grown on malt extract, minimal media and Scots pine sawdust. Bars represent standard deviations. Negative values represent growth stimulation.



(a)



(b)

Figure 2.3. Growth inhibition as a % of the control, of *Serpula lacrymans* H28 (a) and BF050 (b) isolates exposed to the VOCs from nine *Trichoderma* isolates grown on malt extract, minimal media and Scots pine sawdust. Bars represent standard deviations. Negative values represent growth stimulation.

	S. lacrymans strain											
	12C			**Forfar**			**H28**			**BF050**		
Media / ***Trichoderma***	Malt	Minimal	Sawdust	Malt	Minimal	Sawdust	Malt	Minimal	Sawdust	Malt	Minimal	Sawdust
Aureoviride	***	***	***	***	**	**	***	**	***	***	**	***
FY	***	-	**	***	***	***	***	**	***	***	-	**
Harzianum 25	***	-	**	**	-	-	***	*	*	*	-	-
Harzianum 206040	***	*	-	***	***	***	***	-	*	***	**	**
Koningii	**	-	-	**	-	-	*	-	*	**	*~	-
Polysporum	***	**	***	***	*	**	***	**	**	***	**	**
Pseudokoningii	-	-	-	-	-	-	-	-	-	*	*~	-
Viride 70	**	**	*	*	*~	-	-	-	-	**	*~	*
Viride 110	**	***	***	***	**	**	**	***	***	***	*	**

~ represents significant stimulation

Key :

*	$P \leq 0.05$
**	$P \leq 0.01$
***	$P \leq 0.001$
-	$P > 0.05$

Table 2.1. Statistical significance (*p*-values) of levels of growth inhibition in *S. lacrymans* strains after exposure to VOCs from *Trichoderma* species.

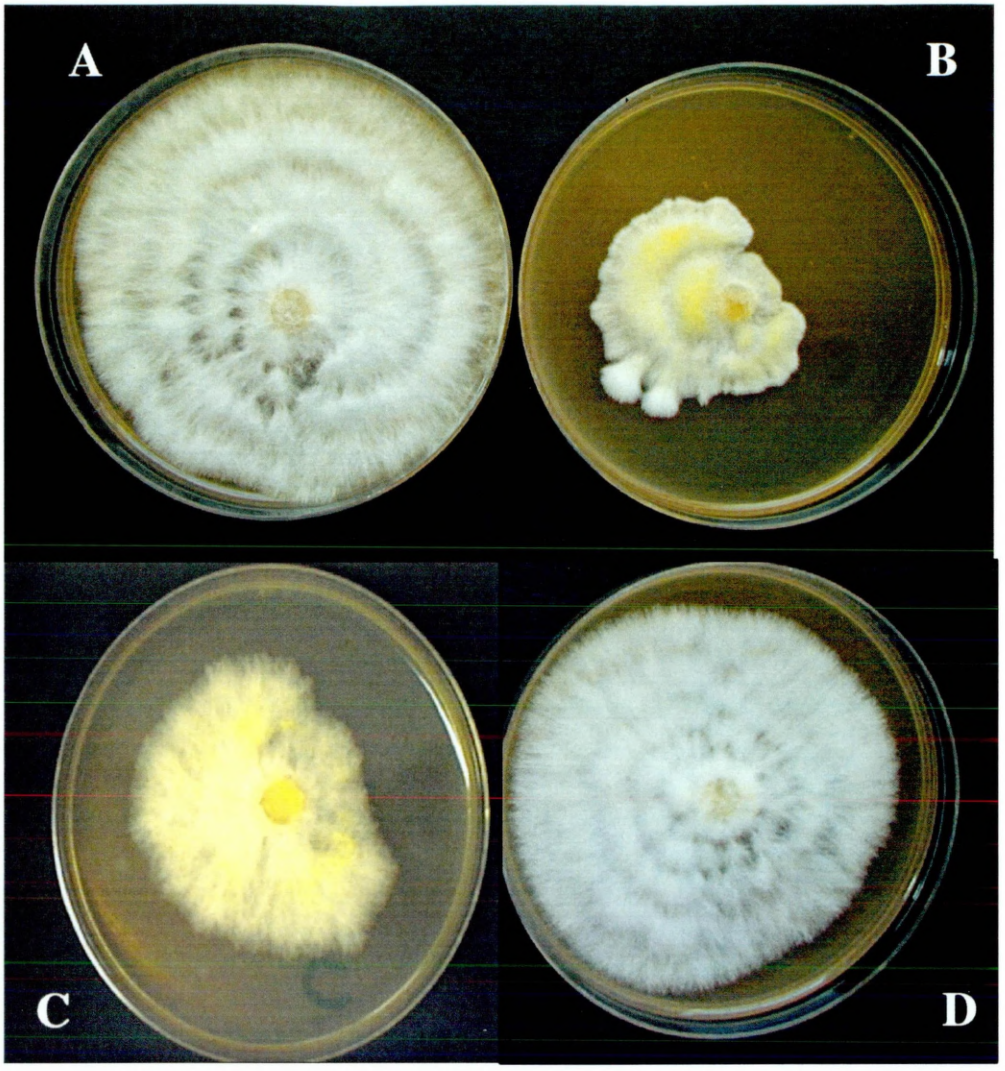


Figure 2.4. Colonies of *S. lacrymans* BF050 demonstrating differing levels of inhibition after exposure to control (A), *T. polysporum* (B), *T. viride* 110 (C) and *T. pseudokoningii* (D).

2.3.2. Extracellular enzyme activity of *S. lacrymans* colony

As the *S. lacrymans* mycelium grew over the Cellulose azure sprinkled on the agar a red colouration was released into the agar and could be seen on the filter paper on the bottom of the gel (Fig.2.5). The results show that cellulase production by *S. lacrymans* was unaffected by the volatiles produced by any of the *Trichoderma* isolates on malt extract agar. Cellulase was produced by all cultures of *S. lacrymans* including controls and cultures that were exposed to the *Trichoderma* VOCs. Tyrosinase was not produced in any of the cultures of *S. lacrymans*, including the cultures that were inhibited by the *Trichoderma* isolates. The effects of VOCs produced by the *Trichoderma* isolates on the peroxidase production of the *S. lacrymans* cultures can be seen in Table 2.2. Peroxidase production was detected in all the cultures of *S. lacrymans* including the control plates and plates that had been exposed to inhibitory *Trichoderma.* The control cultures and isolates where VOCs produced less inhibition of growth such as *T. koningii* IMI 54693 and *T. pseudokoningii* T64 only showed peroxidase production around the core of the *S. lacrymans colony* and not elsewhere on the mycelium. All *Serpula* isolates exposed to *T. polysporum, T. aureoviride*, *T. harzianum* 206040 and FY *Trichoderma* however displayed an intense brown colour (positive for peroxidase production) around the core and spreading out over the entire mycelium of the cultures (Fig. 2.6). It should be noted that these *Trichoderma* isolates also displayed the greatest inhibition of *S. lacrymans* cultures. This was also true for *S. lacrymans* BF050 and Forfar exposed to *T. viride* 110 and *S. lacrymans* 12C and H28 exposed to *T. harzianum* 25. *S. lacrymans* 12C and H28 exposed to the volatiles produced by *T. viride* T110 and *S. lacrymans* exposed to *T. harzianum* 25 on malt extract agar only showed peroxidase production around the core (Table 2.2). Again, this mirrors the levels of growth inhibition with BF050 and Forfar being inhibited to a greater extent by VOCs from *T.*

viride 110 than either 12C or H28 on malt agar and vice versa for the volatiles produced by *T. harzianum* 25 (Fig.2.2 a,b & 2.3 a,b).

***Trichoderma* Isolate**	***S. lacrymans* Isolate**			
	12C	**Forfar**	**BF050**	**H28**
Control	X	X	X	X
T. aureoviride 91968	XX	XX	XX	XX
FY *Trichoderma*	XX	XX	XX	XX
T. harzianum T 25	XX	X	X	XX
T. harzianum IMI 206040	XX	XX	XX	XX
T. koningii IMI 54693	X	X	X	X
T. polysporum IMI 206039	XX	XX	XX	XX
T. pseudokoningii T64	X	X	X	X
T. viride T70	X	X	X	X
T. viride T110	X	XX	XX	X

Key:
X – enzyme release detected
XX – increased enzyme production

Table 2.2. Peroxidase enzyme detected in cultures of *S. lacrymans* exposed to VOCs from *Trichoderma*

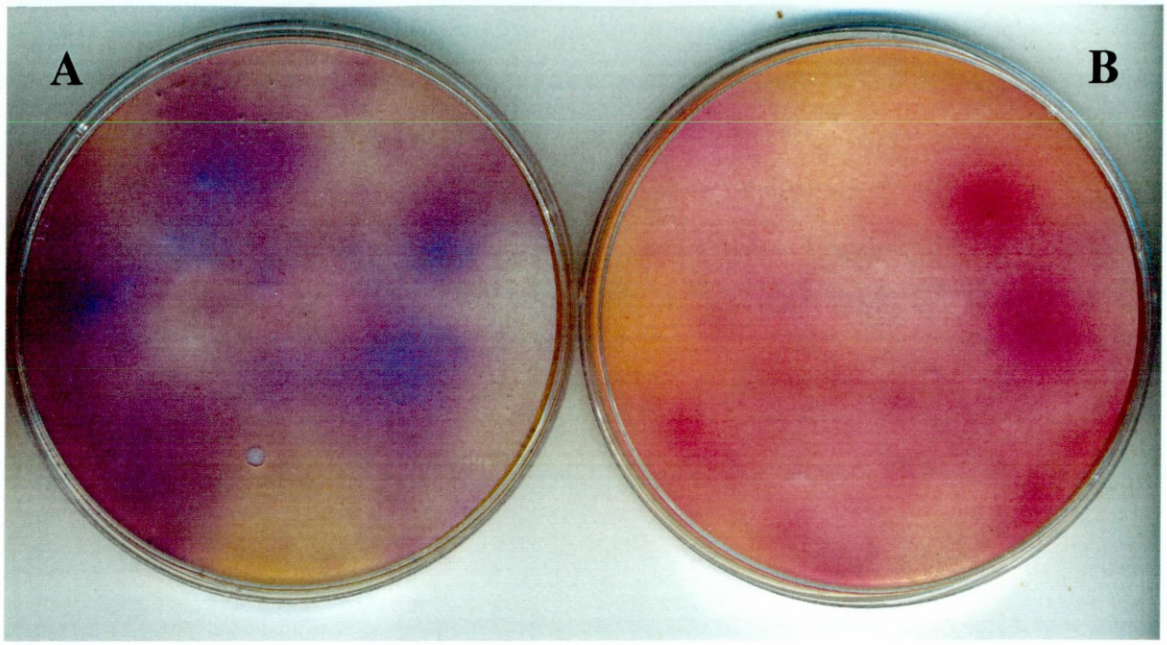


Figure 2.5. Plates showing filter paper on the bottom of malt extract agar sprinkled with cellulose azure before inoculation (A) and after incubation for 7 days with *S. lacrymans* isolate (B). The red colouration is positive for Cellulase activity.

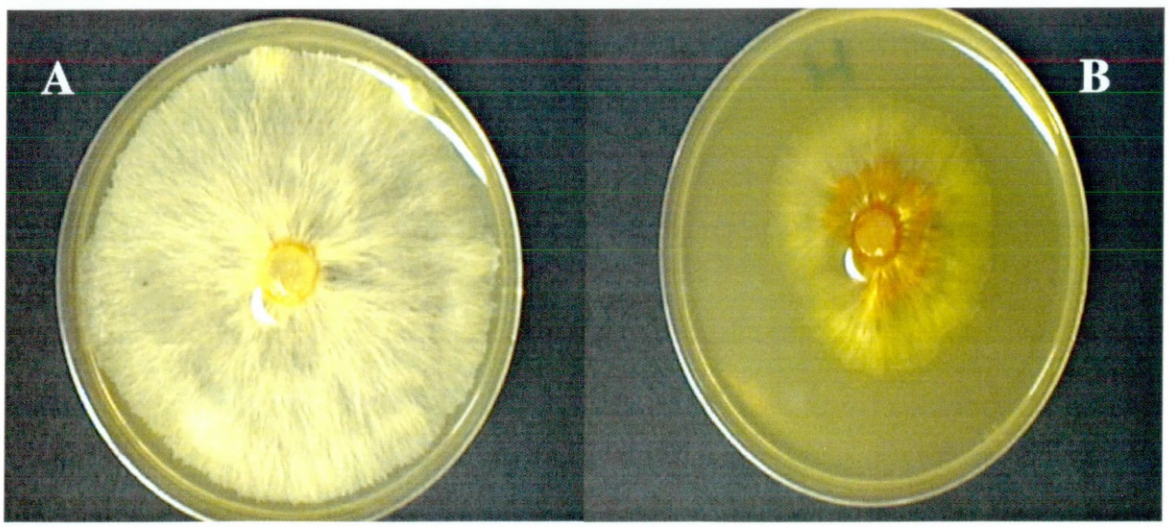


Figure 2.6. (A) *S. lacrymans* H28 exposed to VOCs from *T. pseudokoningii* showing peroxidase production only around the core of the colony. (B) *S. lacrymans* H28 exposed to VOCs from *T. aureoviride* showing peroxidase spreading from the core over the culture.

2.4. Discussion

The results presented here demonstrate that the composition of the growth media has a major effect on the volatile inhibition of *S. lacrymans* by *Trichoderma* species with generally greater inhibition being produced when the *Trichoderma* isolates were cultured on a nutrient rich medium. This is in agreement with various authors who have shown the importance of nutrient composition on the effectiveness of biological control by *Trichoderma* spp. (Srinivasan *et al.*, 1992, Score and Palfreyman 1994; Wheatley *et al.*, 1997). While greater levels of inhibition were produced on the malt extract agar, the volatiles produced were fungistatic rather than fungicidal. There were significant levels of inhibition produced by certain *Trichoderma* isolates when grown on the sawdust and if *Trichoderma* spp. are to be successful as biocontrol agents of dry rot it is important that significant levels of inhibition are produced on the substrate on which they must be active *in situ*. *S. lacrymans* isolates consistently produced a yellow pigmentation when the levels of inhibition by the *Trichoderma* VOCs were greater than 50%. The production of such pigments has been linked to the formation of melanin or melanin-like compounds that could protect hyphal structures from antagonistic fungi (Rizzo *et al.*, 1992). Score and Palfreyman (1994) reported that dual plate interactions between *S. lacrymans* and *Trichoderma* spp. resulted in the mycelium changing colour from white to yellow then brown.

The results also demonstrate that VOCs production was dependent on the *Trichoderma* isolate. Over all three media, the same 2 *Trichoderma* isolates (*T. polysporum* and *T. aureoviride*) consistently produced significant levels of inhibition and one isolate (*T. pseudokoningii*) generally produced no significant levels of inhibition. Interestingly *T. viride* T110 gave higher levels of inhibition when grown on sawdust rather than malt media for *S. lacrymans* H28 and 12C. This could be

because *T. viride* T110 is producing different volatiles when grown on the sawdust or producing certain inhibitory volatiles in greater amounts. Experimentation to identify the active VOCs that are responsible for the inhibition of the *S. lacrymans* is presented in chapter 3. Such compounds could be used in chemical fumigation of wooden structures or as a basis for the selection of *Trichoderma* strains for biological control purposes. Previous work by Wheatley *et al.* (1997) identified five VOCs, acetone, 2-methyl-1-butanol, heptanal, octanal and decanal, produced by *Trichoderma*. These VOCs were implicated in the inhibition of the four wood decay fungi, *Neolentinus lepideus* (Fr:Fr.) Redhead and Ginns (FPRL 7G), *Postia placenta* (Fr) M. Lars et Lomb (FPRL 280), *Gloeophyllum trabeum* (Pers:Fr) Murr. (FPRL 108 N) and *Trametes versicolor* (L.:Fr.) Pilat. (FPRL 28G). Humphris *et al.*, (2001) tested four of these five compounds, acetone, 2-methyl-1-butanol, heptanal and octanal against the four wood decay fungi and found that the two aldehydes, heptanal and octanal, were effective in inhibiting all four fungi.

There were also differing sensitivities between the isolates of *S. lacrymans*, which was most notable on the malt extract agar. On this substrate, H28 was the least susceptible to the *Trichoderma* VOCs and Forfar was the most susceptible. It may be that the Himalayan isolate is more tolerant to environmental changes so has shown greater developed resistance than the other 3 isolates when grown on the malt extract rather than its natural substrate. White *et al.* (1997) have reported previously that wild Himalayan isolates are better suited to growing in a fluctuating or more stressful external environment and that building isolates have adapted for growth within the environmentally narrow building niche.

It may be possible to develop a biological control strategy for *S. lacrymans* by either enhancing or inhibiting the production of specific enzymes by the decay fungus. In this experiment, the production of three enzymes, tyrosinase, peroxidase and cellulase was examined. VOC production by *Trichoderma* did not stimulate the production of tyrosinase by *S. lacrymans* isolates with no colour reaction appearing in any of the combinations of *Trichoderma* and *Serpula* isolates. The function of tyrosinase has been linked to morphogenesis and pigmentation (Jolivet *et al.*, 1998; Flurkey *et al.*, 1995; Griffith *et al.*, 1994). The results of this experiment indicate that tyrosinase was not produced by *S. lacrymans* in either control plates or plates exposed to the VOCs produced by *Trichoderma*. Since yellow pigmentation was often detected in plates of *S. lacrymans* that had been inhibited it is unlikely that tyrosinase plays a role in this pigment production. Score *et al.* (1997) also concluded that tyrosinase was not involved in pigment production by *S. lacrymans* after he found that the production of tyrosinase by *S. lacrymans* was inhibited in confrontation experiments with *Trichoderma* species on malt extract where yellow and brown pigmentation was seen. Zare-Mavain and Shearer (1998) demonstrated that tyrosinase production was dependent on the medium used, with only 10% of freshwater lignicolous fungi producing tyrosinase on malt extract media whereas 45% of the strains of fungi produced this enzyme on cornmeal agar. The use of malt extract agar in this study may therefore account for the lack of production of this enzyme by *S. lacrymans*. Or it may be that tyrosinase production is linked to soluble metabolites released into the media, which could not occur during the volatile mediated interactions.

Peroxidase enzymes have been implicated in the production of highly toxic compounds that are antifungal in nature and in the formation of different types of mycelia and pigment production (Velazhahan and Vidhyasekaran.1994). Score and Palfreyman

(1994) detected peroxidase release throughout the entire colony of *S. lacrymans* but most strongly at the interaction zone during dual plate cultures. These authors speculated that peroxidase may be linked to the formation of mycelial fans in *S. lacrymans* or is linked to the formation of pigments in an attempt to resist invasion. Increased peroxidase production was detected in all *S. lacrymans* cultures that were inhibited by the VOCs from *T. polysporum* IMI 206039, *T. aureoviride* IMI 91968, *T. harzianum* IMI 206040 and FY *Trichoderma* on malt extract agar and, in addition, *S. lacrymans* BF050 and Forfar when exposed to *T. viride* T110 on malt extract agar and in H28 and 12C when exposed to *T. harzianum* 25. All these cultures were inhibited by 50% or more and displayed yellow pigmentation, suggesting that increased peroxidase production is linked to increased pigmentation associated with a stress reaction in the fungus.

Wood degradation by brown rot fungi is reported to include enzymatic and non-enzymatic processes (Highley, 1987). This study has shown that *S. lacrymans* produces cellulase after only 7 days incubation and that cellulase was produced continuously by the *S. lacrymans* isolates and was not affected by the volatiles produced by *Trichoderma*. If the non-enzymatic oxidative agent, which is involved in the early stages of the depolymerisation of cellulose and renders the cyrstalline cellulose more susceptible to enzymatic enzymes, is also be unaffected by *Trichoderma* volatiles, then this suggests *S. lacrymans* would still be capable of degrading cellulose even if its growth was inhibited. However, the level of cellulase produced would be much lower due to the decrease in biomass of *S. lacrymans* caused by the *Trichoderma* VOCs.

CHAPTER 3: Identification of inhibitory VOCs

3.1. Introduction

Microorganisms produce a wide range of VOCs (Mackie & Wheatley, 1999; Evans, 1994; Wheatley *et al.*, 1997) and the identification of specific active compounds can be difficult. Various authors, however, have examined *Trichoderma* isolates and identified possible active compounds. Dennis and Webster (1971b) reported that some *Trichoderma* spp., especially isolates of the *T. viride* species group were found to produce volatile components inhibitory to the growth of other fungi. The active isolates were all characterised by a definite "coconut" smell. They found that *Trichoderma* isolates produced a range of volatile metabolites, which had different effects on different test fungi and tentatively identified acetaldehyde as one inhibitory compound from *T. viride.* Collins and Halim (1972) examined the volatile compounds of strains of *T. viride* and identified 6-pentyl-pyrone as the major coconut aroma constituent. Claydon *et al.* (1987) reported that 6-pentyl-pyrone, produced by 2 strains of *T. harzianum*, had inhibitory effects against a wide range of fungi.

Bruce *et al.* (2000) reported the effects of subtle changes to the amino acid composition of the growth medium on the production of VOCs by *T. aureoviride* and the consequent inhibition of wood decay fungi. The *T. aureoviride* was grown on a minimal media containing phenylalanine, glutamine, arginine or all three. The types and proportions of VOCs produced by *T. aureoviride* were dependent on the amino acid composition of the media on which the fungus was growing. Although *T. aureoviride* produced inhibitory compounds on all four media types, the medium containing only arginine was associated with the highest levels of inhibition of the wood decay fungi and phenylalanine the lowest. The results implicated two aldehydes (nonanal and decanal) in the inhibition of the decay fungi. Wheatley *et al.* (1997) reported that the composition of growth media had a major effect on the type and amount of VOCs

produced by *Trichoderma* isolates and identified five compounds implicated in the inhibition of four wood decay fungi. Their work demonstrates that the VOC profile produced is dependent not only on the media type used but also the strain of *Trichoderma*. Humphris *et al.* (2001) tested four of the five compounds identified by Wheatley *et al.* (1997), acetone, 2-methyl-1-butanol, heptanal and octanal, against four wood decay fungi and found that the two aldehydes, heptanal and octanal were effective in inhibiting all four fungi over a range of concentrations, down to ppm level.

The aim of this chapter was to collect the VOCs produced by three *Trichoderma* isolates selected on the basis of the results presented in chapter 2 and examine the changes in the VOC profiles when grown on different media and to identify the compound or compounds involved in the inhibition of *S. lacrymans*.

3.2. Materials and methods

3.2.1. Collection of VOCs

To identify the active VOC or VOCs involved in the inhibition of *S. lacrymans*, a system was designed to trap and identify the VOCs produced by three of the *Trichoderma* isolates selected from the VOC mediated interactions (Chapter 2, fig. 2.1). These three isolates were selected on the following basis:
T. aureoviride 91968 generally gave the greatest levels of inhibition over the range of media and *S. lacrymans* isolates, whereas *T. pseudokoningii* T64 generally gave the poorest levels of inhibition when examining the results for all *S. lacrymans* isolates on all media. *T. viride* T110 was unusual compared with the other isolates tested in that it gave greater levels of inhibition when grown on sawdust and minimal media rather than malt for *S. lacrymans* H28 and 12C (Figs. 2.2a,b & 2.3a,b, Chapter 2, section 2.3.1).

Aluminium trays containing either 150ml of 3% malt extract agar, 150mls of 1.5% minimal media (Chapter 2, section 2.2.2) or Scots pine sawdust (24g sawdust/48ml water) were all inoculated in the centre with either a single core (5mm in diameter) of *T. aureoviride*, *T. pseudokoningii* or *T. viride* 110. All treatments were performed in triplicate and included uninoculated controls of each media type. The cultures on the malt extract agar and the minimal media were incubated at 25°C for 5 days and then placed into separate closed fermentation vessels (Fig. 3.1) and returned to the incubator for another 2 days. In order to give the *Trichoderma* isolates time to colonise the sawdust media and to replicate the conditions in chapter 2 (Section 2.2.2) the cultures on the Scots pine sawdust were incubated for 12 days and then placed into separate closed fermentation vessels and returned to the incubator for another 2 days. After incubation, pressurized purified air was circulated through the vessels at 50ml min^{-1} for

2 hours; as the air was circulated it passed through collection tubes containing 0.4g of the chromatographic adsorbent Tenax-GR (Phase Separations, UK). Before entering the fermentation vessels the pressurized air was passed through 2 Puritubes (Phase Separations, UK), one containing activated charcoal and the other containing molecular sieve to remove any contaminating VOCs and water from the air. Puritubes and collection tubes were conditioned prior to use in a helium stream at a flow rate of 25ml min^{-1} at 180°C for the Puritubes and 240°C for the Tenax-GR collection tubes for 24 hours. This pre-conditioning activated the adsorbent and removed any background contaminants. After 2 hours, collection tubes were removed from the fermentation vessels and dried by reverse flushing with nitrogen at a flow rate of 20ml min^{-1} for 15 min.

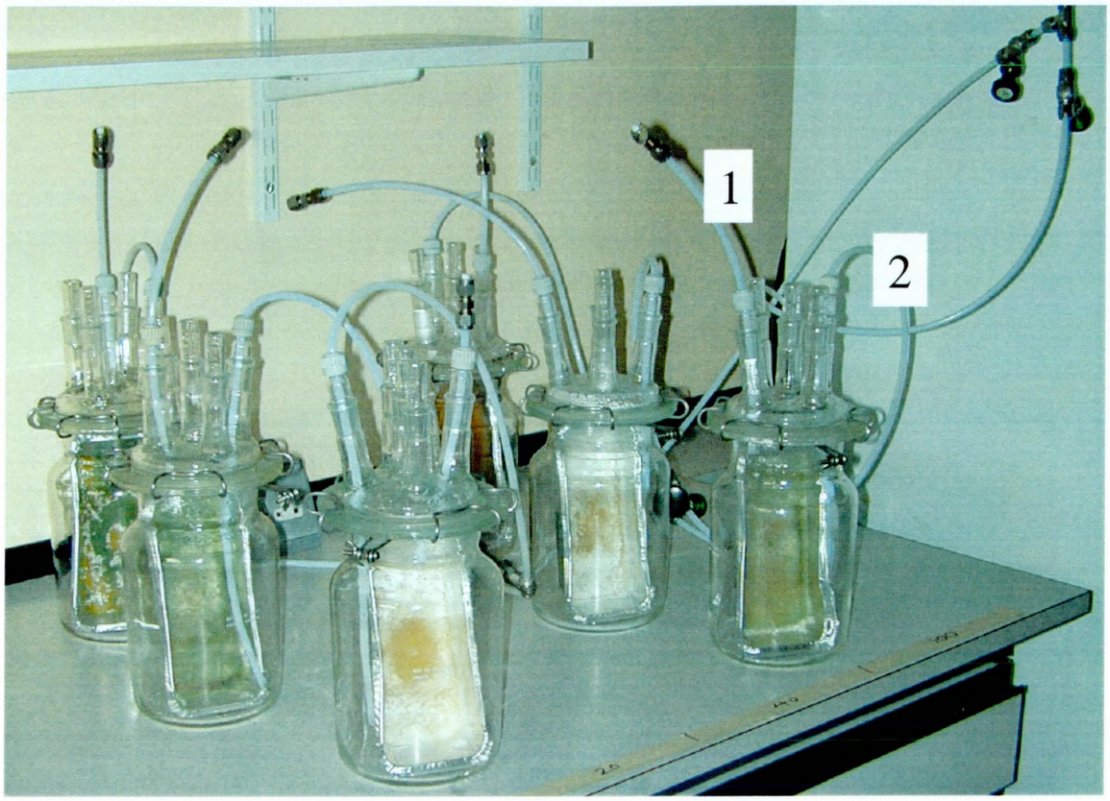


Figure 3.1. Cultures of *Trichoderma* grown on malt extract agar and sealed in fermentation vessels. After being sealed for 2 days, a collection tube was screwed on top of tube 1 and then pressurized purified air was circulated into the vessels from tube 2 which then circulated from the vessels and through the collection tube.

3.2.2. Analysis of VOCs

The volatile compounds adsorbed were analysed on an integrated system consisting of a Perkin Elmer ATD 50 automated thermal desorber connected to a Hewlett Packard 5890 gas chromatograph interfaced to a VG Trio 1000 quadrupole mass spectrometer. Automated thermal desorption took place in 2 stages. Adsorbent tubes were heated to 130°C for 15 minutes with helium gas passing through them, the released VOCs were cryofocussed on a Tenax-TA cold trap held at -25°C. The temperature was then rapidly increased to 240°C, which transferred the VOCs to a DB1701 capillary column as a concentrated sample pulse. The carrier gas flow was $1ml^{-1}$ with an inlet split ratio of 7.5:1. The GC column temperature was programmed from 40°C to 200°C at 5°C/min and then held isothermally for 20 min. After separation on the column, the components passed into the quadrupole mass spectrometer that scanned over the range 20-400 amu at a rate of 1 second per scan. Ionization was by electron impact at 70eV with a trap current of 150μA. Source temperature was held at 200°C. An empty tube was desorbed between each sample to minimise any possible memory effects in the GC column. Each analysis took approximately 80 min.

The GC-MS data was recorded on a computer using Masslab version 1.3 software package (Thermofinnigan, UK) and integrated chromatographic peak areas derived from the total ion current signal. Compounds were identified by comparison of their mass spectra with those in the NIST/NBS (Thermofinnigan, UK) mass spectral databases.

3.2.3. Principal component analysis

Principal component analysis was used to summarize the relationships between the VOCs, after standardising each VOC to have mean 0 and standard deviation 1. The principal component analysis extracts linear combinations of the VOCs, which account for as much of the variation as possible. Thus, the data may be represented in a small number of dimensions. This allows identification of inter-relationships between the production of individual VOCs by *Trichoderma* isolates when grown on each of the three media. A detailed description on the use of biplots can be found in Phillips & McNicol (1986).

3.3. Results

3.3.1. Collection of VOCs

A representative chromatogram from the GC-MS analysis is shown in figure 3.2. The x-axis represents the time required for the compound to pass through the GC-MS, where larger compounds will be retained in the column for longer. The y-axis of the graph is a measure of the total peak areas, where the compound with the largest area is assigned 100% and values for all other compounds in the sample are calculated relative to this. Once Masslab had integrated the individual peaks, the values of the peak areas were given as a percentage of the total peaks integrated. Each peak was then individually compared with a reference library of compounds and where a fit value of greater than 900 was seen (maximum of 1000) the identification was considered to be acceptable. In cases where fits lower than 900 were given, the accuracy of the match was considered by comparing the mass spectra, the respective base peaks and the molecular masses of the unknown and the software identified standard (Fig.3.3). For comparative identification purposes the masses 28 (nitrogen) and 32 (oxygen) were excluded, as these are likely to be air forced into the column during desorption. Peaks that were identified as siloxane derivatives and Chlorofluoro carbonates (CFCs) were assumed to be background contaminants and were therefore discarded.

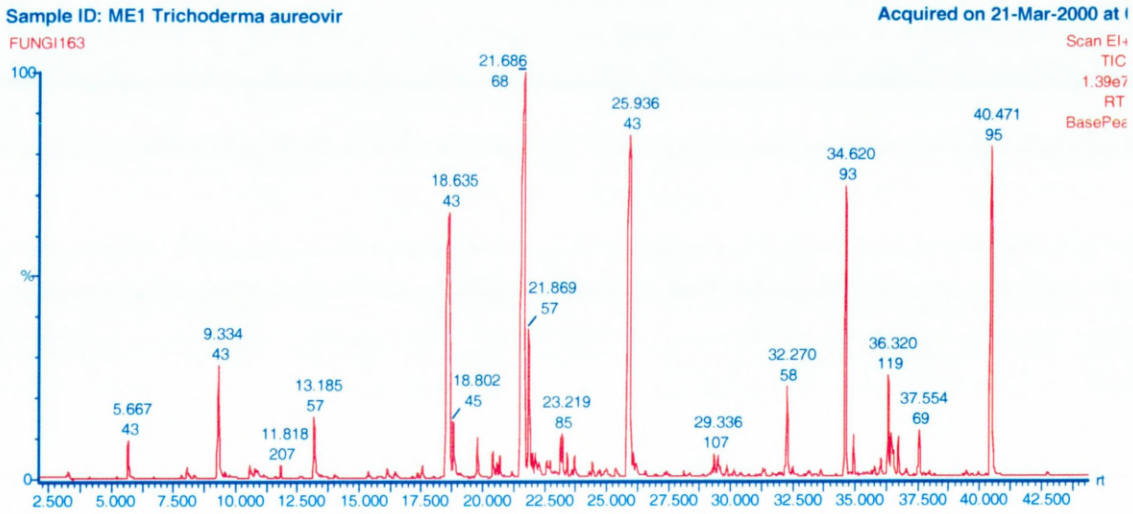


Figure 3.2. Representative spectra from GC-MS showing peaks of all the compounds produced by *Trichoderma aureoviride* 91968 when grown on malt extract agar. The x-axis represents retention time in minutes and the y-axis of the graph is a measure of the total peak areas, where the compound with the largest area is assigned 100% and values for all other compounds in the sample are calculated relative to this.

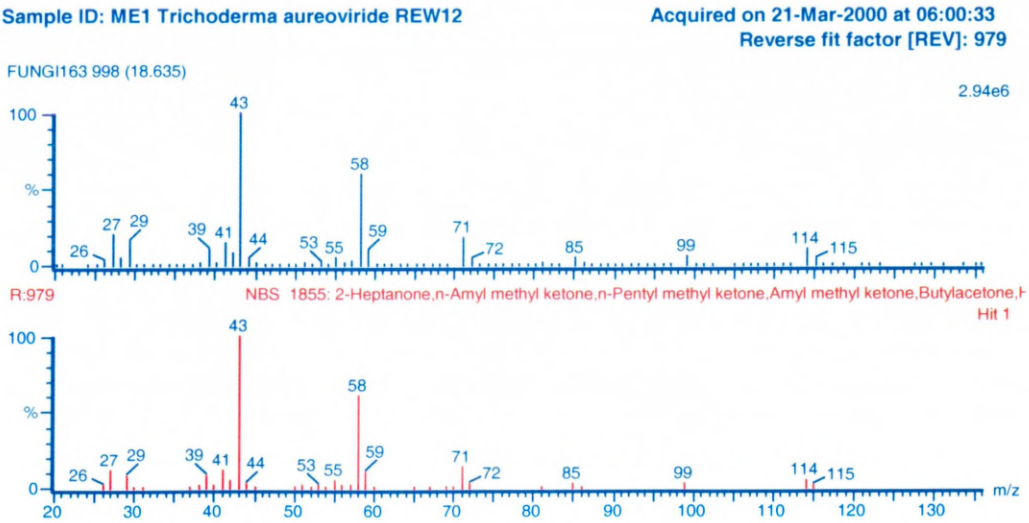


Figure 3.3. Representative fingerprint of unknown peak at retention time 18.635 from *Trichoderma aureoviride* (in blue) identified as 2-heptanone by the mass spectral database libraries (in red). The names after 2-heptanone are other names that 2-heptanone is known by.

3.3.2. Analysis of VOCs

After correction for the levels of volatiles produced in the uninoculated control samples a total of 41 different volatile compounds were identified that were produced by the three *Trichoderma* grown on the three media (Tables 3.1-3.3). Any compounds produced by both the uninoculated control samples and the test samples but in larger amounts by the controls were eliminated from the selection. Two of the compounds, 2-pentanone and 2-nonanone, were produced by both the media blanks and the media inoculated by *T. viride* and *T. aureoviride,* but in larger amounts by the inoculated media. In this instance the amounts produced by the media blanks were subtracted from the amounts produced by the inoculated media. Comparison of the VOC profiles show that both the *Trichoderma* isolate and the medium used have an effect on the range and quantity of VOCs produced. Over all 3 media *T. aureoviride* consistently produced the greatest number of compounds and *T. pseudokoningii* the lowest, with *T. aureoviride* and *T. viride* consistently producing most VOCs on the malt media. *T. aureoviride* and *T. viride* share more compounds in common with each other rather than with *T. pseudokoningii*, except when grown on minimal media. On this media the 2 isolates with the most compounds in common are *T. aureoviride* and *T. pseudokoningii*. *T. viride* produced compounds 2-pentanone (number 6), 2-heptanone (number 19) and 2-nonanone (number 27) on all media. *T. aureoviride* produces compounds 2-heptanone (number 19), 3-octanone (number 22), benzene (number 23) and 2-nonanone (number 27) on all media. There is no compound produced by *T. pseudokoningii* on all 3 media. It is reasonable to assume that *T. aureoviride* and *T. viride* would have more compounds in common with each other rather than with *T. pseudokoningii* as this strain generally didn't produce any significant levels of inhibition when compared with the control.

Table 3.1. VOCs from *T.pseudokoningii*, *T. viride* and *T. aureoviride* when grown on malt media.

	MALT MEDIUM								
	T. pseudokoningii			*T. viride*			*T. aureoviride*		
	Replicate			Replicate			Replicate		
COMPOUND	1	2	3	1	2	3	1	2	3
1 Ethanol	-	-	-	-	-	-	-	-	-
2 Propyl alcohol	51.4	64.7	53.8	-	-	-	-	-	-
3 1heptene	-	-	-	14.1	6.4	1.4	-	-	-
4 Isobutanol	-	-	-	-	-	-	5.9	4.9	8.8
5 2methyl1propanol	-	-	-	-	-	-	-	-	-
6 2pentanone, ethyl acetone	-	-	-	2.8	2.4	1.6	0.3	0.4	0.5
7 2pentanol	-	-	-	-	-	-	-	-	-
8 Disulfide	-	-	-	-	-	-	-	-	-
9 1.4cyclohexadiene	-	-	-	2.3	7.8	5.5	-	-	-
10 Butyl methyl2pentanone	-	-	-	-	-	-	-	-	-
11 3methyl1butanol	-	-	-	-	-	-	-	-	-
12 2methyl1Butanol	34.9	28.2	29.5	-	-	-	2.2	2.0	2.2
13 1,3Octadiene	3.3	2.0	3.9	-	-	-	-	-	-
14 2hexanol	-	-	-	-	-	-	-	-	-
15 1nonene	-	-	-	3.8	4.6	6.8	0.3	0.4	0.5
16 1hexene	-	-	-	-	-	-	-	-	-
17 Benzene, 1,2 dimethyl	-	-	-	1.9	1.7	1.3	0.3	0.2	0.9
18 4methyl2hexanone	-	-	-	-	-	-	-	-	-
19 2heptanone	-	-	-	38.0	32.4	25.3	23.5	27.0	25.6
20 2heptanol	-	-	-	-	-	-	1.9	0.9	2.6
21 Bicyclo[3.1.1]heptane	-	-	-	-	-	-	-	-	-
22 3octanone	-	-	-	-	-	-	5.2	4.7	10.0
23 Benzene	-	-	-	-	-	-	0.9	0.7	0.8
24 2octanol	-	-	-	-	-	-	-	-	-
25 Hexanal	-	-	-	-	-	-	-	-	-
26 Cyclopropane	5.2	4.2	6.8	-	-	-	-	-	-
27 2nonanone	-	-	-	24.3	26.4	28.6	31.3	35.8	33.8
28 1undecanol	-	-	-	-	-	-	-	-	-
29 2decanone	-	-	-	-	-	-	-	-	-
30 bicyclo[3.1.0]hexan2ol	-	-	-	-	-	-	-	-	-
31 bicyclo[3.1.1]heptan3one	-	-	-	-	-	-	-	-	-
32 Phenol, pcresol	4.3	1.7	4.6	2.5	0.4	-	0.6	1.3	0.9
33 bicyclo[3.1.1]hept2ene	-	-	-	-	-	-	-	-	-
34 2dodecanone	-	-	-	-	-	-	3.8	2.5	2.6
35 Naphthalene,octahydro	-	-	-	-	4.3	4.6	-	-	-
36 propanoic acid	2.0	-	2.2	-	-	-	-	-	-
37 1,3cyclohexadiene	-	-	-	-	-	-	1.6	0.8	0.8
38 cyclohexene	-	-	-	-	-	-	1.4	1.7	0.8
39 1,5cyclodecadiene	-	-	-	5.2	9.5	14.7	-	-	-
40 spiro[5.5]undeca1,8diene	-	-	-	5.6	5.0	1.2	-	-	-
41 Naphthalene, decahydro	-	-	-	-	-	-	21.5	17.4	1.0

Values refer to the percentage of the total 41 compounds after correction for uninoculated controls.

Table 3.2. VOCs from *T.pseudokoningii*, *T. viride* and *T. aureoviride* when grown on minimal media.

	MINIMAL MEDIUM								
	T. pseudokoningii			*T. viride*			*T. aureoviride*		
	Replicate			Replicate			Replicate		
COMPOUND	1	2	3	1	2	3	1	2	3
1 Ethanol	-	38.5	16.4	-	-	-	15.6	23.2	17.5
2 Propyl alcohol	-	1.5	1.1	-	-	-	3.0	3.6	6.7
3 1heptene	-	-	-	-	-	-	-	-	-
4 Isobutanol	-	-	-	-	-	-	-	-	-
5 2methyl1propanol	-	17.5	2.5	-	-	8.3	15.4	13.2	11.4
6 2pentanone, ethyl acetone	-	-	-	-	-	-	-	-	-
7 2pentanol	-	-	-	-	-	-	-	-	-
8 Disulfide	-	1.2	1.1	15.7	28.2	13.8	-	-	-
9 1.4cyclohexadiene	-	-	-	-	-	-	-	-	-
10 Butyl methyl2pentanone	-	-	-	-	-	-	-	-	-
11 3methyl1butanol	-	22.8	31.2	-	-	-	7.4	4.1	6.5
12 2methyl1Butanol	-	18.5	21.2	-	-	-	5.4	2.9	5.7
13 1,3Octadiene	-	-	-	-	-	-	-	-	-
14 2hexanol	-	-	-	-	-	-	-	-	-
15 1nonene	-	-	-	-	-	-	-	-	-
16 1hexene	-	-	-	1.2	2.3	-	-	-	-
17 Benzene, 1,2 dimethyl	-	-	-	-	-	-	-	-	-
18 4methyl2hexanone	-	-	-	-	-	-	-	-	-
19 2heptanone	-	-	-	45.5	43.4	34.9	26.3	23.2	25.0
20 2heptanol	-	-	-	-	-	-	-	-	-
21 Bicyclo[3.1.1]heptane	-	-	-	-	-	-	-	-	-
22 3octanone	-	-	-	-	-	-	6.2	7.8	7.7
23 Benzene	-	-	-	-	-	-	-	-	-
24 2octanol	-	-	-	-	-	-	-	-	-
25 Hexanal	-	-	-	-	-	-	-	-	-
26 Cyclopropane	-	-	-	-	-	-	-	-	-
27 2nonanone	-	-	-	17.2	7.6	1.5	1.4	7.9	1.0
28 1undecanol	-	-	-	-	-	-	-	-	-
29 2decanone	-	-	-	-	-	-	-	-	-
30 bicyclo[3.1.0]hexan2ol	-	-	-	-	-	-	-	-	-
31 bicyclo[3.1.1]heptan3one	-	-	-	-	-	-	-	-	-
32 Phenol, pcresol	-	-	-	-	-	-	-	-	-
33 bicyclo[3.1.1]hept2ene	-	-	-	-	-	-	-	-	-
34 2dodecanone	-	-	-	-	-	-	-	-	-
35 Naphthalene,octahydro	-	-	-	1.5	8.4	5.3	-	-	-
36 propanoic acid	-	-	-	-	-	-	-	-	-
37 1,3cyclohexadiene	-	-	-	-	-	-	-	-	-
38 cyclohexene	-	-	-	-	-	-	-	-	-
39 1,5cyclodecadiene	-	-	-	5.2	1.2	9.8	-	-	-
40 spiro[5.5]undeca1,8diene	-	-	-	13.8	-	18.6	-	-	-
41 Naphthalene, decahydro	-	-	-	-	-	-	1.4	15.1	9.7

Values refer to the percentage of the total 41 compounds after correction for uninoculated controls.
There are no values for *T. pseudokoningii* replicate 1 as sample was lost during GC-MS

Table 3.3. VOCs from *T.pseudokoningii*, *T. viride* and *T. aureoviride* when grown on sawdust media.

	SAWDUST MEDIUM								
	T. pseudokoningii			*T. viride*			*T. aureoviride*		
	Replicate			Replicate			Replicate		
COMPOUND	1	2	3	1	2	3	1	2	3
1 Ethanol	-	-	-	-	-	-	-	-	-
2 Propyl alcohol	-	-	-	-	-	-	-	-	-
3 1heptene	-	-	-	-	-	-	-	-	-
4 Isobutanol	-	-	-	-	-	-	-	-	-
5 2methyl1propanol	-	-	-	-	-	-	-	-	-
6 2pentanone, ethyl acetone	74.0	45.5	69.6	19.8	24.5	-	22.3	28.4	21.6
7 2pentanol	-	-	-	1.7	1.5	-	2.0	-	3.9
8 Disulfide	-	-	-	-	-	-	-	-	-
9 1.4cyclohexadiene	-	-	-	-	-	-	-	-	-
10 Butyl methyl2pentanone,	13.1	9.8	1.7	-	-	-	1.6	-	2.3
11 3methyl1butanol	-	-	-	-	-	-	-	-	-
12 2methyl1Butanol	-	-	-	-	-	-	-	-	-
13 1,3Octadiene	-	-	-	-	-	-	-	-	-
14 2hexanol	-	-	-	2.9	2.7	-	1.6	-	3.0
15 1nonene	-	-	-	-	-	-	-	-	-
16 1hexene	-	-	-	-	-	-	-	-	-
17 Benzene, 1,2 dimethyl	-	-	-	-	-	-	-	-	-
18 4methyl2hexanone	-	-	-	-	-	-	1.9	-	1.9
19 2heptanone	-	-	-	45.7	42.9	-	35.7	37.7	2.4
20 2heptanol	-	-	-	11.1	1.9	-	-	-	-
21 Bicyclo[3.1.1]heptane	5.4	1.3	7.2	-	-	-	-	-	-
22 3octanone	-	-	-	-	-	-	0.9	1.2	0.9
23 Benzene	-	-	-	-	-	-	1.2	-	1.2
24 2octanol	-	-	-	3.9	4.3	-	-	-	-
25 Hexanal	-	-	-	-	-	-	3.9	4.7	6.8
26 Cyclopropane	-	-	-	-	-	-	-	-	-
27 2nonanone	-	-	-	1.5	8.9	-	25.6	28.8	24.3
28 1undecanol	-	8.4	6.5	-	-	-	-	-	-
29 2decanone	-	-	-	-	-	-	0.9	-	1.5
30 bicyclo[3.1.0]hexan2ol	-	1.7	6.6	-	-	-	0.8	-	1.3
31 bicyclo[3.1.1]heptan3one	-	-	-	-	1.4	-	1.4	-	1.5
32 Phenol, pcresol	-	-	-	-	-	-	-	-	-
33 bicyclo[3.1.1]hept2ene	7.4	15.7	-	2.9	1.8	-	1.6	-	1.8
34 2dodecanone	-	-	-	-	-	-	-	-	-
35 Naphthalene,octahydro	-	-	-	-	-	-	-	-	-
36 propanoic acid	-	-	-	-	-	-	-	-	-
37 1,3cyclohexadiene	-	-	-	-	-	-	-	-	-
38 cyclohexene	-	-	-	-	-	-	-	-	-
39 1,5cyclodecadiene	-	-	-	3.7	1.8	-	-	-	-
40 spiro[5.5]undeca1,8diene	-	-	-	-	-	-	-	-	-
41 Naphthalene, decahydro	-	-	-	-	-	-	-	-	-

Values refer to the percentage of the total 41 compounds after correction for uninoculated controls.
There are no values for *T. viridie* replicate 3 as sample was lost during GC-MS

3.3.3. Principal component analysis

The results of the principal component analysis can be appropriately represented as biplots (Fig. 3.4 a-e). The biplots summarise data on the treatment (*Trichoderma* species × media) and volatile production simultaneously. The influence of a particular *Trichoderma* species when grown on a single medium type on the production of a particular volatile can be assessed from the biplot. The smaller the distance from the origin to a perpendicular projection from the replicates of any one treatment onto a line from the origin to the VOC, the greater the influence of that media type on the production of the individual VOC by the *Trichoderma*. Figure 3.4a summarises the relationship between the treatments without showing the volatile compounds. Figure 3.4b shows the separation of the VOCs produced by all nine treatments (*Trichoderma* species × media). Due to the excessive amount of information on this one bi-plot, the information was separated into production of VOCs by each individual *Trichoderma* isolate on the three media to produce three more bi-plots (Fig. 3.4c-e). Figure 3.4c separates the VOCs produced by *T. aureoviride* grown on all 3 media and Figures 3.4d and 3.4e separate the VOCs produced by *T. viride* 110 and *T. pseudokoningii* on all 3 media respectively.

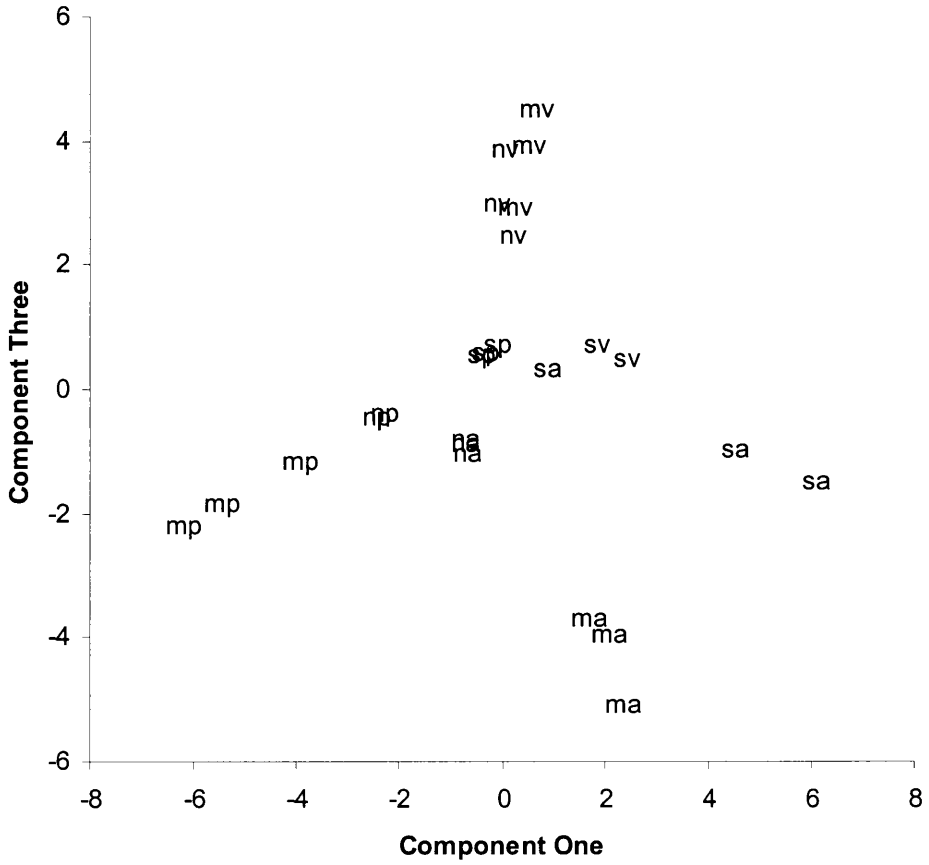


Figure 3.4a. Principal component analysis (PCA) biplot summarises the relationship between the treatments.

Key: ma - *T. aureoviride* grown on malt extract agar; **mv** – *T. viride* grown on MEA; **mp** – *T. pseudokoningii* grown on MEA; **na** - *T. aureoviride* grown on minimal media; **nv** – *T. viride* grown on MM; **np** – *T. pseudokoningii* grown on MM; **sa** - *T. aureoviride* grown on sawdust media; **sv** – *T. viride* grown on sawdust media and sp – *T. pseudokoningii* grown on sawdust media.

The three replicates of each of the nine treatment groups lie close to one another, with the exception of one replicate of *T. aureoviride* grown on sawdust (sa). This demonstrates that the reproducibility of each treatment is very good. The one replicate of *T. aureoviride* grown on sawdust which is separated from the other two replicates may be caused by the variability of constituents in wood such as amino acid content or may indicate a small amount of heartwood may have been mixed in with the sapwood when the sawdust was produced. Nayagam (1987) found that even in the outer 10 growth rings of wood, amino acid content varied between the rings. All the treatment groups, except for *T. viride* grown on malt and minimal media, are separated from one another which indicates that the range of VOCs released are clearly influenced by the both the media type and the *Trichoderma* isolate. The fact that the treatment groups for *T. viride* grown on malt and minimal media overlap indicates that they produce a similar range of compounds; there is however no single compound, which is produced by all antagonists on all media (Tables 3.1-3.3). The range of VOCs produced will represent the metabolic conversion by the organisms of the media constituents. This may well explain why the *Trichoderma* isolates grown on the sawdust media are grouped together away from the other treatments, as the aromatic compounds produced are due to the sawdust and are not detected in any of the other treatments (Tables 3.1-.3.3). One treatment group that is most clearly separated out from the other groups is *T. aureoviride* grown on malt media. This is the treatment that gives the greatest levels of inhibition, so clearly VOCs located close to this will be of particular interest. The other treatment group that is also more separated than some of the other groups is *T. aureoviride* grown on sawdust. The compounds produced around this group or more importantly between this group and *T. aureoviride* on malt (ma) are of particular significance for the selection of *Trichoderma* strains as biocontrol agents of dry rot *in*

situ. Any potential biocontrol strain must also produce any compounds identified from the most inhibitory treatment when grown on sawdust.

Figure 3.4b separates the VOCs produced by all nine treatments (*Trichoderma* species × media). The influence of a particular treatment on the production of an individual VOC can by assessed from this bi-plot. The closer the individual VOC is located to the replicates of a particular treatment the greater the influence of that treatment on its production. The numbers on the bi-plots refer to the numbers given to the individual VOCs in Tables 3.1-3.3. The numbers around the treatment that gives the greatest levels of inhibition (*T. aureoviride* grown on malt media) that are of interest are 4, 22, 34, 37, 38 and 41. By referring back to Tables 3.1-3.3 it can be seen that number 4 is isobutanol; number 22 is 3-octanone; number 34 is 2-dodecanone; number 37 is 1,3 cyclohexadiene; number 38 is cyclohexene and number 41 is naphthalene.

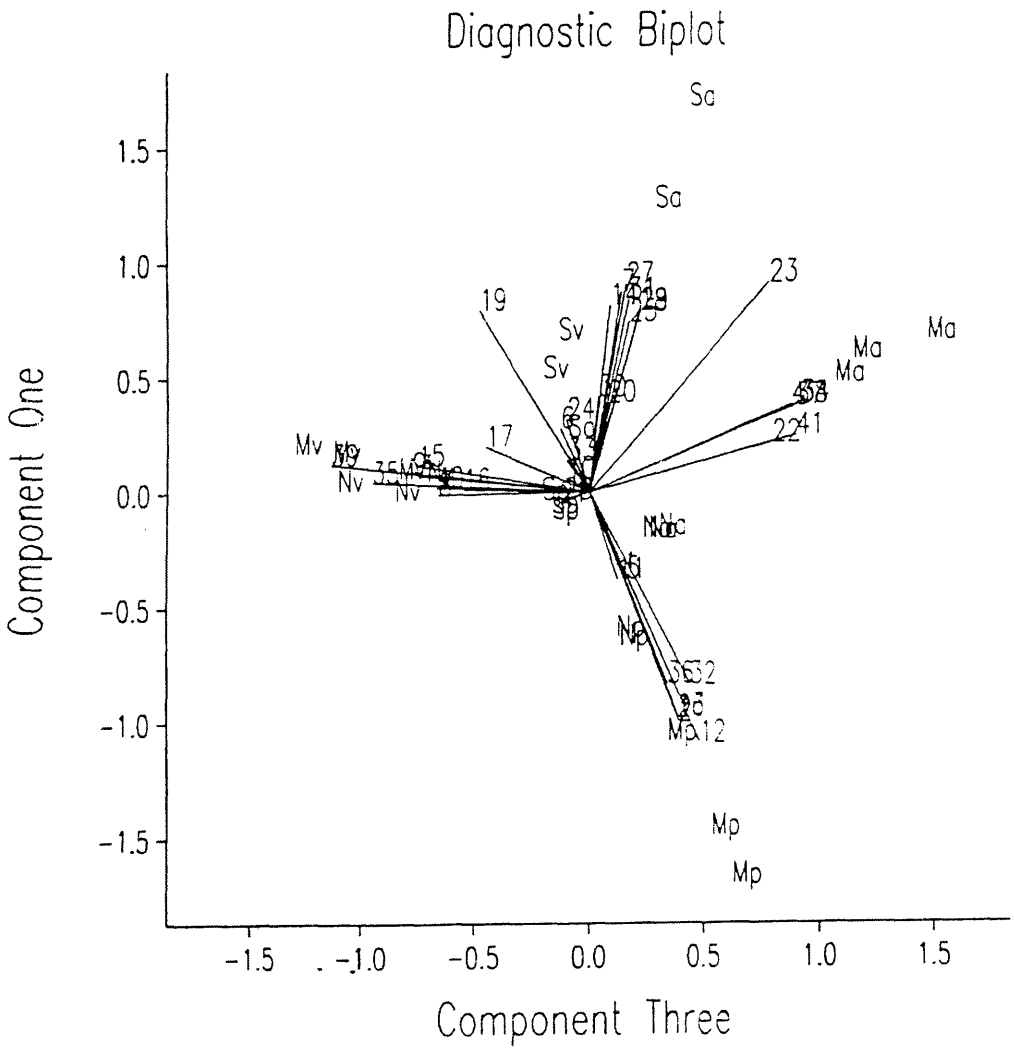


Figure 3.4b. Principal component analysis biplot separating the VOCs produced by all nine treatments (*Trichoderma* species × media).

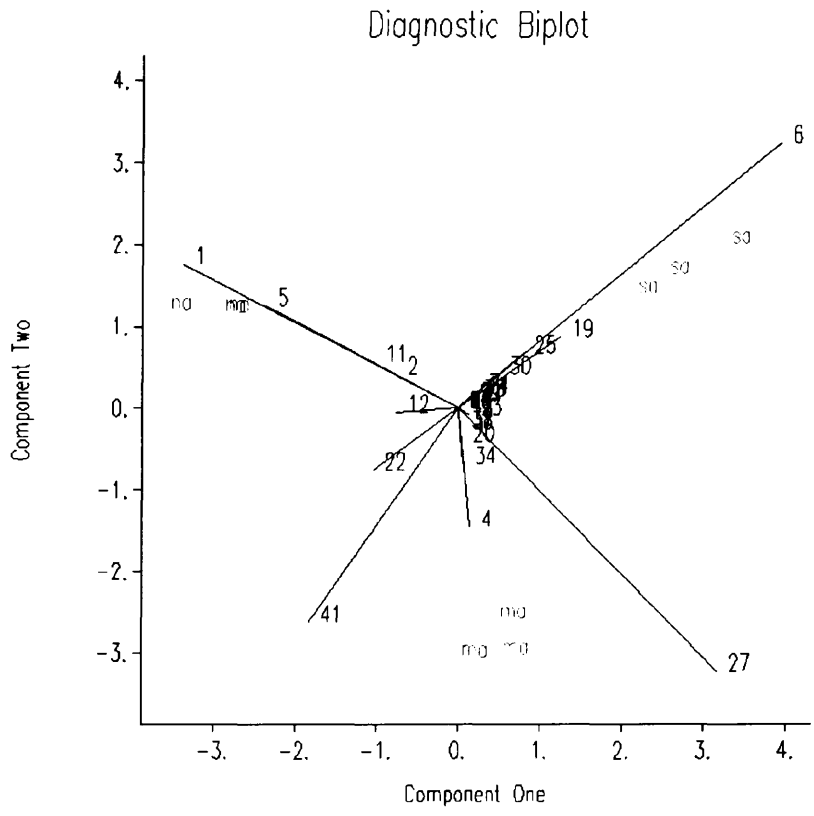


Figure 3.4c. PCA biplot separating VOCs produced by *T. aureoviride* grown on malt extract (ma), minimal media (na) and sawdust (sa).

All treatment conditions represented on this bi-plot produced significant levels of inhibition of the target decay fungus (Fig. 3.5) therefore this bi-plot defines inputs rather than inhibition and demonstrates the difference that nutrient composition has on the compounds produced. As before, compounds 4, 22, 34 and 41 are still clearly significant. Compounds 37, 38 and 23 are difficult to see as they are closer to the origin; this is because compounds 37 and 38 are produced in much smaller amounts than for example, compound 4 or 41 by *T. aureoviride* on malt agar (Table 3.1). Due to the fact that this bi-plot only has three treatment groups, compounds produced in large amount seem much more significant, however compounds produced in small amounts

may be just as potent inhibitors of target fungi as a compound produced in large amounts. Compound 6 has been highlighted by this bi-plot as significant for treatment sa. However, by looking at Fig. 3.4e it can be seen that this compound is also produced in large amounts by *T. pseudokoningii* when grown on sawdust, so therefore is not important in the inhibition of *S. lacrymans*. This demonstrates that it is important to look at and compare the information on all biplots. Another compound of interest situated between treatments ma and sa is compound 27, 2-nonanone. This compound is produced in significant amounts by *T. aureoviride* on malt media but is also produced on sawdust (Tables 3.1-3.3).

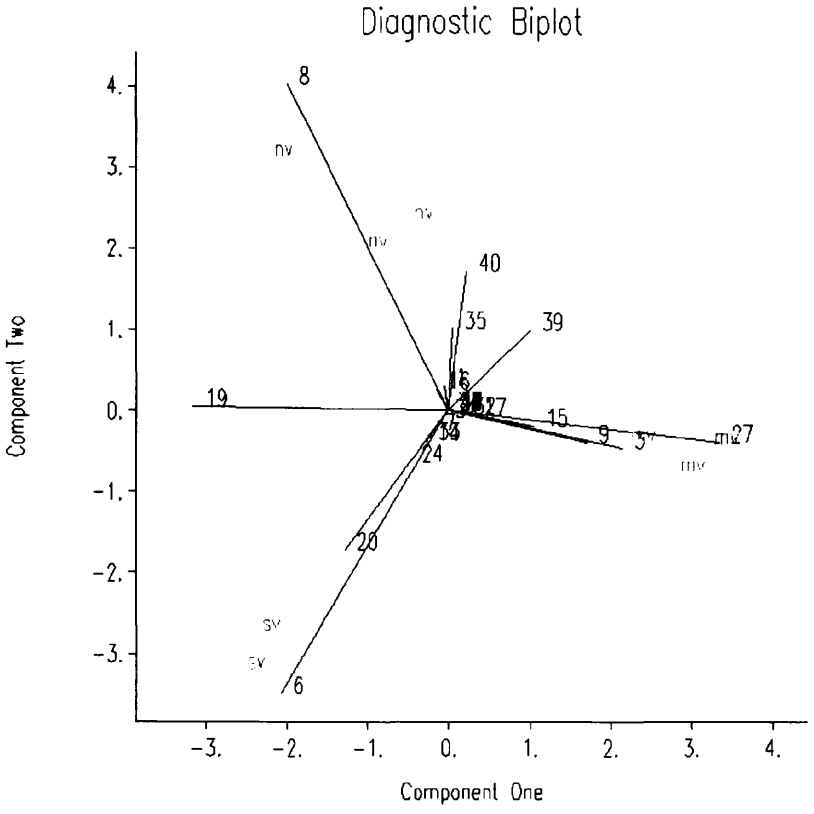


Figure 3.4d. PCA biplot separating VOCs produced by *T. viride* grown on malt extract (mv), minimal media (nv) and sawdust (sv).

T. viride grown on malt (mv) still gave significant inhibitory effects on growth of *S. lacrymans* but was less inhibitory that *T. aureoviride* grown on malt (see Fig. 3.5). Therefore, a compound of interest would be close to the treatment mv but must also be produced by *T. aureoviride* grown on malt (ma) (Fig. 3.4e). Compound number 27, 2-nonanone is significant for mv and also significant for ma (Fig. 3.4e). An interesting anomaly found from the results of chapter 2 (Section 2.3.1) was that *T. viride* gave higher levels of inhibition when grown on sawdust and minimal media rather than malt for *S. lacrymans* H28 and 12C. Therefore any compounds situated between *T. viride* grown on minimal media (nv) and *T. viride* grown on sawdust (sv) are significant. So another compound of interest highlighted on this bi-plot is number 19, 2-heptanone.

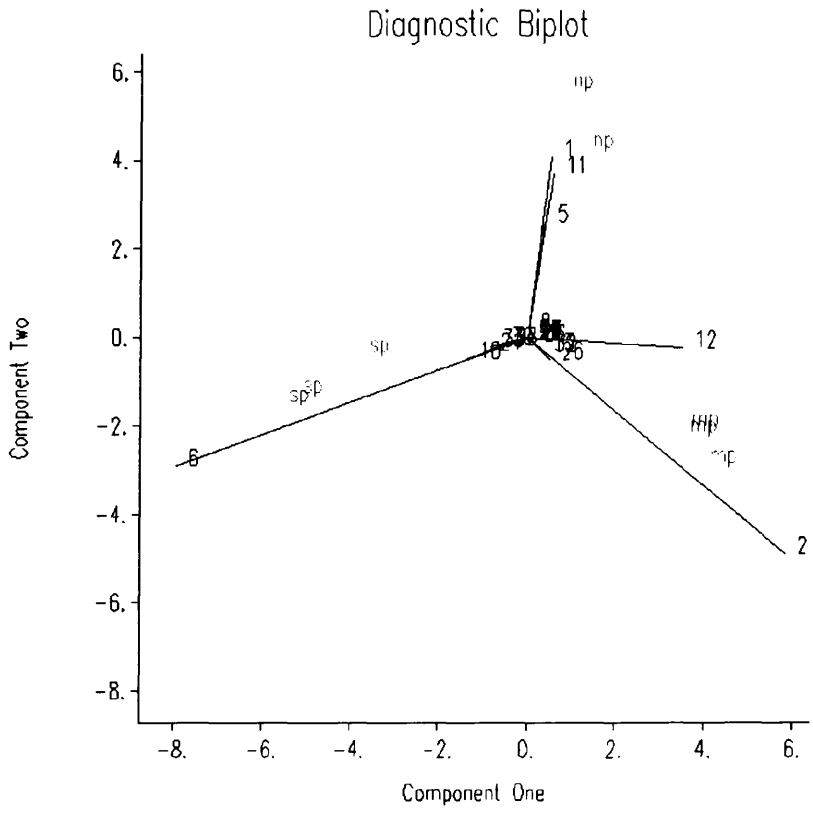


Fig. 3.4e. PCA biplot separating VOCs produced by *T. pseudokoningii* grown on malt extract (mp), minimal (np) and sawdust media (sp).

As can be seen from the graph in Fig. 3.5 volatiles from *T. pseudokoningii* had very little or no inhibitory effect and in some case even stimulated the growth of *S. lacrymans*. Therefore any compounds produced by this fungus are unlikely to contribute to the inhibitory effect. As can be seen from the bi-plot (fig, 3.4e), very few compounds have been pulled out by the treatment groups and the majority of compounds lie around the origin of the bi-plot. Therefore these treatments only influenced the production of a small number of compounds none of which can be considered to be significant in the inhibition. The majority of stimulation of *S. lacrymans* growth was when the *T. pseudokoningii* was grown on minimal media; therefore, compounds 1, 5 and 11 may be significant in the stimulation of *S. lacrymans*.

It is interesting that all significant compounds, except number 6, (1, 2, 5, 11 and 12) highlighted on this bi-plot are alcohols (Tables 3.1-3.3).

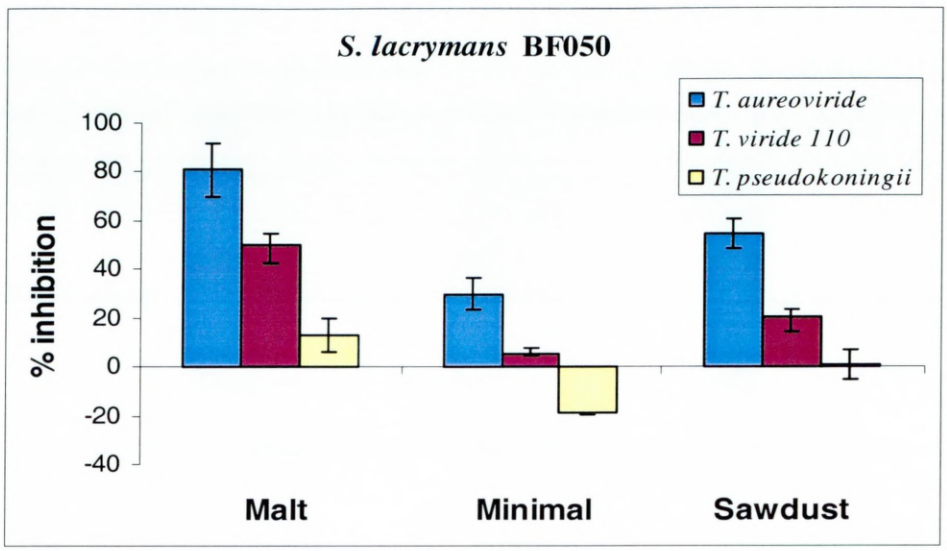


Figure 3.5. Growth inhibition (%) of *S. lacrymans* BF050 exposed to VOCs from *T. aureoviride*, *T. viride* and *T. pseudokoningii* on malt extract, minimal and sawdust media. Results taken from chapter 2 showing levels of inhibition in relation to VOCs produced by the *Trichoderma* isolates.

In summary, the compounds of interest produced only by *T. aureoviride* on the malt media are 2-dodecanone (number 34); 1,3 cyclohexadiene (number 37); Cyclohexene (number 38) and Isobutanol (number 4). Naphthalene (number 41) was produced by *T. aureoviride* on the malt and minimal media but only in small amounts on the minimal media. There was one compound 3-octanone (number 22) produced by *T. aureoviride* on all 3 media but in greater amounts on the malt media which is also implicated in the inhibition of *S. lacrymans*. 2-nonanone (number 27) was produced by both *T. aureoviride* and *T. viride* on all three media with both isolates producing a small amount on minimal media and *T. aureoviride* producing greater amounts than *T. viride* on the malt and sawdust media. Both *T. aureoviride* and *T. viride* produced 2-heptanone (number 19) on all 3 media, with *T. viride* producing larger amounts on the minimal media and sawdust.

3.4. Discussion

Under constant conditions all microbial species produce a reproducible profile of volatile organic compounds (VOCs) that can be identified (Zechman and Labows, 1985; Giudici *et al.,* 1990; Bruce *et al.,* 2000). These profiles can be used as a means of identifying microorganisms growing on building materials or wood (Sunesson *et al.*, 1996; Bjurman *et al.*, 1997.) Both Miller *et al.* (1988) and Strom *et al.* (1994) have used 2-heptanone as a microbial marker to identify mould fungi in buildings. In wood VOCs released by resident microorganisms may build up and become toxic to other organisms that colonise the wood (Bruce *et al.* 1996). As microbial activity is greatly influenced by environmental factors, similarly production of these volatile compounds will also be affected by the dynamics of the whole ecosystem. The range and diversity of VOCs produced is affected by the nutrients that are available (Fiddaman and Rossall, 1993; Larsen and Frisvad 1994; Bruce *et al.,* 2000), the environmental temperature (Tronsmo and Dennis 1978), the species (Sunesson *et al.*, 1995; Wheatley, *et al.,* 1997) and age of the microorganism (Bruce *et al.,* 1996).

The analysis of VOC production by the three *Trichoderma* isolates implicated 8 compounds: 2-dodecanone (number 34); 1,3 cyclohexadiene (number 37); cyclohexene (number 38); isobutanol (number 4); naphthalene (number 41); 3-octanone (number 22); 2-nonanone (number 27) and 2-heptanone (number 19); that may be involved in the inhibition of *S. lacrymans.* The biplots have shown that the test system is robust and the results reproducible and that it can highlight compounds from those treatments that are known to produce high levels of inhibition. Bruce *et al.* (1996) found that the age of *T. aureoviride* cultures influenced the levels of inhibition of decay fungi and also affected the VOC profile with 7-14 day old cultures of *T. aureoviride* giving greatest inhibition of decay fungi corresponding to a major switch from alcohol to aldehyde and ketone

production. Therefore in this study the samples were collected from cultures of *Trichoderma* within that age range and interestingly here again has resulted in half of the compounds being identified as ketones. The results from chapter 2 (section 2.3.1) demonstrated that *T. pseudokoningii* in some cases caused stimulation of *S. lacrymans* rather than inhibition (see Fig. 3.5, *T. pseudokoningii* stimulated *S. lacrymans* BF050 when grown on minimal media). From the results of the bi-plot showing VOC production for *T. pseudokoningii* grown on the 3 media (Fig. 3.4e) it can be seen that 5 of the 6 compounds (1, 2,5,11 and 12), which were significant for those treatments, were alcohols. Humphris *et al.* (2001) reported that an alcohol (2-methyl-1-butanol) also stimulated the growth of the basidiomycete *P. placenta* at low concentrations.

If *Trichoderma* spp. are to be applied as biocontrol agents for the remedial treatment of dry rot in buildings then compounds produced under the most inhibitory conditions (*T. aureovirde* grown on malt media) must also be produced on the sawdust. Therefore, of the 8 compounds identified 3 appear to have a greater significance, 3-octanone, 2-nonanone and 2-heptanone, as they were all produced on both the malt media and sawdust. Both 2-nonanone and 2-heptanone were produced in large amounts on the sawdust whereas 3-octanone was produced in relatively small amounts. However, the amount of the volatile produced does not determine the compounds potency and in substrates such as wood where cell lumina can allow slow gaseous diffusion, VOCs released by the *Trichoderma* spp. may build up and become toxic to the *S. lacrymans*. The greater amounts of 2-heptanone produced on the sawdust and minimal media by *T. viride* might explain the higher levels of inhibition seen for *S. lacrymans* H28 and 12C compared with the *T. aureoviride* on the same media. The fact that only *S. lacrymans* 12C and H28 showed this increase in inhibition is probably due to species specificity with these two isolates being more susceptible to 2-heptanone. On the minimal media,

T. aureoviride and *T. pseudokoningii* shared more compounds in common whereas on the other 2 media *T. aureoviride* and *T. viride* had more compounds in common. Two of the compounds produced by *T. aureoviride* but not by *T. pseudokoningii* on the minimal media were interestingly 2-nonanone and 2-heptanone, which supports the implication of these 2 compounds in the inhibition of growth of *S. lacrymans*.

The results from the volatile identification experiments suggest that there is not one compound involved in the inhibition of growth of *S. lacrymans* but rather two or more compounds cause the inhibitory effect. Testing the individual compounds or a combination of the compounds and examining the levels of inhibition of *S. lacrymans* could determine this. Bruce *et al.* (2000) indicated two aldehydes (nonanal and decanal) in the inhibition of the decay fungi and Dennis and Webster, (1971b) tentatively identified acetaldehyde as one inhibitory compound from *T. viride.* Both authors however concluded that *Trichoderma* isolates probably produce more than one active volatile metabolite. It may be possible to use any of the other 6 compounds or indeed any of the 8 compounds identified in this chapter as chemical fumigants for wooden structures. Specific VOCs previously identified as compounds that inhibited the growth of 4 wood decay fungi including heptanal and octanal (Wheatley *et al.*, 1997) have been shown to be effective over a range of concentrations, down to ppm level (Humphris *et al.*, 2001). Even if any of the identified VOCs in this study were also effective at this low concentration it is unlikely that they could ever be used as part of a chemical fumigation system to control *S. lacrymans* in wood due to the toxicity of the compounds. However this would require further testing of the chemicals to determine their toxicity levels.

It seems most likely that application of *Trichoderma* strains with increased ketone production may provide an environmentally acceptable treatment for short-term protection of wood *in situ* in buildings. Microorganisms produce volatiles that have an unpleasant odour (Larsen and Frisvad, 1994) and excessive growth of microorganisms is implicated in health problems in buildings, known as sick house syndrome (Platt *et al.*, 1989). *Trichoderma* used *in situ* in buildings could have adverse health effects even though the volatiles are produced in low concentrations, which would need to be taken into account if *Trichoderma* species are to be applied as biological control agents in houses. So it is likely that biological control with *Trichoderma* spp. would be limited to situations where human contact with treatment areas would not be for a long period of time, such as the remedial treatment of dry rot or preserving buildings which are falling into disrepair.

The work in this chapter has shown that volatiles from *T. aureoviride* can still give significant levels of inhibition of *S. lacrymans* when grown on sawdust, a more realistic environment rather than malt or minimal media. The levels of inhibition may even be higher when in wood as *Trichoderma* would be closer in contact with the *Serpula* than in the volatile mediated interactions used in this study (see chapter 2). The results from the studies in chapter 2 also indicated that *Serpula* strains inhibited by VOCs were found to produce increased levels of peroxidase enzymes and suggested that this might be associated with an increased stress response by the decay fungus. Therefore, it could be that one or more of the compounds implicated in the inhibition are also responsible for the increased levels of peroxidase production. What has not been determined is whether *Trichoderma* spp. produces a different profile of compounds, which may be more or less inhibitory, when grown on different wood species due to the ranges of chemical extractives from different woods and within different parts of wood such as

the heartwood and sapwood. It is also possible and indeed likely, that *Trichoderma* spp. could produce a different range of compounds in the presence of competitor organisms and none of them could be the compounds identified in this study. Therefore, for *Trichoderma* spp. to be effective as a biological control agent *in situ* it is necessary to also examine VOC profiles produced after *Trichoderma* spp. has been exposed to *S. lacrymans*. Bruce *et al.* (1991) have previously shown that *Trichoderma* can survive and continue to inhibit wood decay fungi for at least seven years within wooden poles although further work is needed to determine the longevity of protection that the *Trichoderma* isolate would provide against dry rot in wood.

CHAPTER 4: Effect of VOCs on protein expression by *S. lacrymans*

4.1. Introduction

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of proteins allows dissociation of proteins into their individual polypeptide subunits, which migrate through polyacrylamide gels in accordance with the size of the polypeptide. The combination of SDS-PAGE with silver staining is a highly sensitive technique allowing the detection of nanogram amounts of protein and by using markers of known molecular weight, it is therefore possible to estimate the molecular weight of unknown polypeptide chains (Sambrook *et al.*, 1989). Protein analysis of soluble proteins extracted from fungal mycelia has been widely used in the identification and classification of fungal isolates including *Penicillium* species (Bent, 1967) and the taxonomy of *Verticillium* species (Milton *et al.*, 1971). SDS-PAGE has also been used to identify species of *Sclerotinia* (Tariq *et al.*, 1985) and different species of *Phytophthora* (Hansen *et al.*, 1986, 1988).

Schmidt & Moreth-Kebernik (1995) found that each species of *Poria* has its own individual fingerprint and electrophoresis differentiates the various species and distinguishes them from other fungi infecting building timbers. Vigrow (1992) compared the mycelium of 14 building isolates of *S. lacrymans* from around the world against the standard test strain FPRL 12C. Comparisons were also made with other morphological forms of this fungus, recently isolated strains of *S. lacrymans* from the field, as well as other basidiomycetes. The results demonstrated that 12 of the 14 building isolates of *S. lacrymans* had visually identical profiles, but other basidiomycetes species showed very few bands in common with *S. lacrymans*. While banding patterns of different morphological forms of *S. lacrymans* and recently isolated strains showed less similarity (Vigrow, 1992), they nevertheless still showed greater

similarity than non-*S. lacrymans* organisms and a basic common banding pattern could be recognized.

When grown under standardised conditions (media, incubation and temperature) highly conserved protein profiles have been seen for *S. lacrymans* isolates (Vigrow *et al.*, 1989). A change in the fungus's environment however is likely to result in a change in the protein profile. This could include growth on different substrates Vigrow *et al.*, 1989), temperature shocks (Sienkiewicz *et al.*, 1997), UV radiation (Friman, 1993) and nitrogen limitation (Gordon and Lilly, 1995). It is likely therefore that when *S. lacrymans* is exposed to other environmental stresses including antagonism from other members of its natural ecosystem, for example VOCs (Wheatley *et al.*, 1997), similar effects may be produced.

The aim of this chapter therefore was to expose selected *S. lacrymans* isolates to VOCs from antagonistic *Trichoderma* species and compare the protein profiles produced under these stressed conditions against profiles from *S. lacrymans* which were not exposed to similar stressed conditions.

4.2. Materials and methods

4.2.1. VOC mediated interactions

Identical tests were set up as described in Chapter 2 section 2.2.2 (Fig. 4.1) except that *Trichoderma* isolates were only grown on 3% malt extract (Oxoid, Hampshire, UK) and the *S. lacrymans* on 5% malt extract (Oxoid)/ 2% agar (Oxoid).

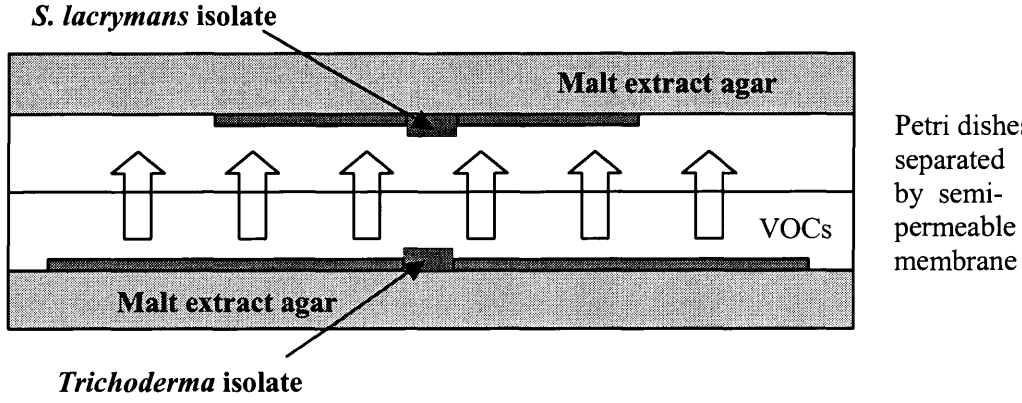


Figure 4.1. Experimental set-up for VOC mediated interactions.

Only three *Trichoderma* isolates were used in this experiment *T. aureoviride* IMI 91968, *T. pseudokoningii* SWIT 64 and *T. viride* T110. These isolates were selected on the following basis:

T. aureoviride IMI 91968 generally gave one of the highest levels of inhibition of all *Trichoderma* isolates tested when grown on the malt extract medium, whereas *T. pseudokoningii* SWIT 64 generally gave no statistically significant levels of inhibition when examining the results for all *S. lacrymans* isolates. *T. viride* T110 was selected because it generally gave an intermediate level of inhibition of around 35-50% on malt extract (See graphs 2.2a,b & 2.3a,b, Chapter 2, section 2.3.1). These strains therefore provided varying levels of inhibition and as previously (Chapter 2, section 2.2.1), 4 *S. lacrymans* isolates were used. Controls were set up for each

S. lacrymans isolate and they contained no *Trichoderma* inocula on the bottom plate. Six replicates were set up for all tests and eight replicates for all controls. All cultures were incubated at 21°C until the growth of the *S. lacrymans* in the controls had reached the edge of the plate (usually 7 days). The complete experiment was then repeated to establish whether banding patterns on SDS-PAGE were consistently reproducible.

4.2.2. Extraction of proteins

Gloves were worn at all times and all procedures were carried out on ice.

After the 7 days incubation at 21°C, the over and under plates were separated and plates containing the *Trichoderma* were discarded. The original inoculation cores were removed and the remaining *S. lacrymans* mycelia were harvested using a spatula to gently remove the mycelia from the agar. Two plates of each treatment were pooled to give one replicate resulting in 3 replicates for each treatment and 4 replicates for each control. All mycelia were washed 3 times in phosphate buffered saline (PBS) containing proteinase inhibitors (Appendix B, 1.1), then frozen in liquid nitrogen and lyophilised overnight (Edwards, UK).

The lyophilised mycelia were ground in liquid nitrogen in a pre-chilled mortar and pestle, using acid purified sand (Fluka, Dorset, UK) as the grinding agent. Once a fine powder was achieved, it was transferred to a 1.5ml Eppendorf and 600µl of ice cold PBS containing the proteinase inhibitors was added. The sample was homogenized by vortexing for 30sec and grinding using a micro-pestle. Samples were spun at 13, 200 rpm for 10 minutes at 4°C in a refrigerated microcentrifuge (Sigma Laboratories, Germany) and the resulting pellet was discarded and the supernatant retained.

4.2.3. Protein concentration

The concentration of protein present in the supernatant was determined using the Bradford Reagent micro-assay following the manufacturer's instructions based on the method of Bradford (1976). The adsorbance of Bovine Serum Albumin protein standards (Sigma, Dorset, UK) of concentrations 125, 250, 500, 750, 1000, 1500 and 2000 μg/ml were read on a MRX Microtitre® plate reader (Dynex Technologies, Sussex, UK) at 595nm in order to generate a protein standard graph against which protein extracts were compared. Aliquots were taken from the supernatant and the samples with more than 150μg/ml of protein were diluted with PBS containing the proteinase inhibitors to ensure all the samples contained this same amount of protein. All aliquots and stocks of supernatant were stored at -20°C.

4.2.4. Preparation of acrylamide gels

All chemicals, electrophoresis grade, were purchased from Sigma (Dorset, UK) unless otherwise stated and ultra-pure distilled water (u-p dH_2O) was always used.

Glass plates, spacers and combs were soaked in 10% Decon® (Decon Laboratories, Sussex, UK) overnight, washed with hot water, rinsed in distilled water and then a final rinse in u-p dH_2O. The plates, spacers and combs were then wiped with 100% ethanol and assembled together according to the manufacturer's instructions. The assembled glass plates were placed into the casting stand and a line marked on the front plate 4cm from the top (Fig. 4.2).

Fifty ml of a 15% resolving gel was prepared to provide enough to produce 2 gels:

u-p dH_2O	18.25ml
1.5M resolving gel buffer (Appendix B, 1.2)	12.50ml
40% Acrylamide	18.75ml
10% SDS	0.50ml

Immediately before pouring the gel 250µl of 10% ammonium persulphate (APS) and 25µl of N,N,N′,N′-Tetramethylethylenediamine (TEMED) was added to the resolving gel mix and mixed thoroughly. The final mixture was pipetted into the glass plates up to the mark, using a 5ml pipette. Water saturated butanol was overlaid onto the freshly poured gel mix to prevent desiccation during polymerization and to obtain a level gel. Once the resolving gel was set (approx. 2 hours) the butanol was poured off and the top of the gel was rinsed using u-p dH_2O and a final rinse in a 1:4 dilution of the resolving gel buffer. The gel could be stored overnight in the fridge by leaving a 1cm layer of the 1:4 dilution of the resolving gel buffer on top of the gel. Once the gel was ready to be used 20ml of a 4.5% stacking gel was prepared (enough for 2 gels):

u-p dH_2O	12.8ml
1.5M stacking gel buffer (Appendix B, 1.3)	5.0ml
40% Acrylamide	2.0ml
10% SDS	0.2ml

Immediately before pouring the gel 100µl of 10% APS and 20µl of TEMED was added to the solution, mixed thoroughly and pipetted on to the top of the resolving gel. The comb was immediately inserted into the solution at an angle to ensure no bubbles formed under the wells and the gel was allowed to set for approximately 40mins. Once set, the comb was removed and the wells rinsed with 1 X electrode buffer (Appendix B, 1.4).

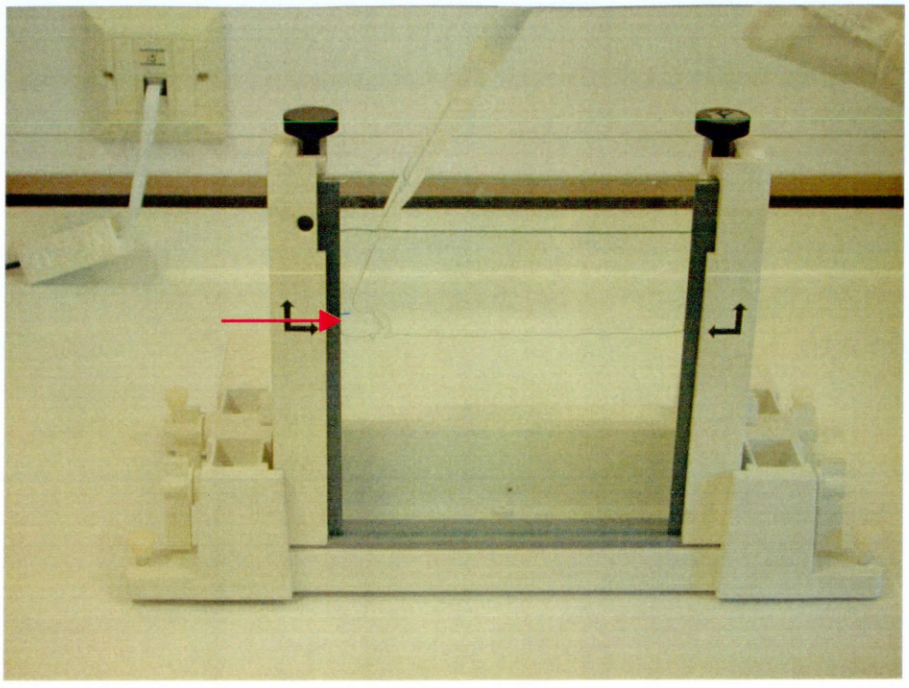


Figure 4.2. Acrylamide gel production set-up. The resolving gel was pipetted up to the mark on glass plate (→) and allowed to set. Once set the stacking gel was pipetted on top.

4.2.5. Sample preparation

The diluted aliquots of *S. lacrymans* isolate were thawed on ice, then mixed in a 1:1 ratio with Laemmli sample buffer (Appendix B, 1.5), boiled for 5 minutes and microcentrifuged at 13, 200rpm for 5 minutes. Forty μl of each sample was loaded into the wells of the gel ensuring that replicate samples were loaded side by side and interspaced with equal lanes of the *S. lacrymans* control. On each gel there were 13 lanes of samples (3 replicate lanes of *S. lacrymans* exposed to *T. pseudokoningii*; 3 replicate lanes of *S. lacrymans* exposed to *T. viride*; 3 replicate lanes of *S. lacrymans* exposed to *T. viride* and 4 replicate lanes of control samples). The final 2 lanes at either side of the gel contained molecular weight markers as described over the page.

4.2.6. Molecular weight markers

Seven molecular weight (MW) markers were used in the Dalton Mark VII-L kit (Sigma, Dorset, UK). Laemmli sample buffer (1.5ml) was added to the lyophilized mixture of the 7 proteins, which was then aliquoted into 40µl samples and stored at -20°C. Before use, the aliquoted marker solution was defrosted on ice, boiled for 5 min and spun for 5 min at 13, 200 rpm before loading 10µl per well on the gels.

4.2.7. Electrophoresis

The equipment used for the electrophoresis was the Protean II xi Cell and an electrophoresis powerpack unit (Bio-rad laboratories, Hertfordshire, UK). All gels were run in 1 X electrode buffer (Appendix B, 1-4) at 40mA for 1 hour until the protein samples had run through the stacking gel; the voltage was then increased to 80mA for approximately 3 hours or until the dye front had reached the base of the gel. Cold running water was used as a cooling system, which ran through the central core of the Protean II xi Cell to prevent thermal band distortion during electrophoresis. Directly after electrophoresis the two plates were separated carefully using a palette knife and the gel was silver stained.

4.2.8. Silver staining

The Bio-rad silver stain plus kit catalogue No. 161-0449 (Bio-rad, Hertfordshire, UK) was made up and used according to the manufacturer's instructions. For best results each step of the procedure required gentle agitation of the gel in solution using a shaker table. The gel was fixed in the fixative enhancer solution for 20min and then rinsed twice in u-p dH_2O, each time for 10 min. The staining solution stained and developed the bands in one step and was left until the desired staining intensity was reached but no

longer than 20min. The staining reaction was stopped by immersing the gel in 5% acetic acid for 15min and then finally rinsing in u-p dH_2O for 5min.

4.2.9. Data analysis

Gel images were scanned using an Epson scanner 1640XL (Getech, Suffolk, UK) and the images processed using GelCompar (Applied Maths, Kortrijk, Belgium). For comparison of results on different gels, the images were aligned with each other using reference positions on the marker lanes. Bands were selected using an automated band search followed by manual adjustment with reference to the original gel, from which the image was scanned. Band based similarity coefficients were calculated according to the Jaccard coefficient. For each couple of lanes the Jaccard coefficient divides the number of corresponding bands by the total number of bands in both lanes. Position tolerance allows the programme to account for minor lane-to-lane variations in band position. Finally, GelCompar dendograms were created using the unweighted pair group method using arithmetic averages (UPGMA) clustering algorithm.

4.3. Results

4.3.1. Protein concentration

Concentrations of estimated protein from each *S. lacrymans* replicate were calculated by extrapolation from the protein standard graph shown in figure 4.3. Typical protein concentrations for *S. lacrymans* controls and samples exposed to VOCs from *T. pseudokoningii* ranged from 750-2300 µg/ml. These were much higher than the samples exposed to VOCs from *T. viride* and *T. aureoviride,* which ranged from 500-1500 µg/ml and 150-400 µg/ml respectively.

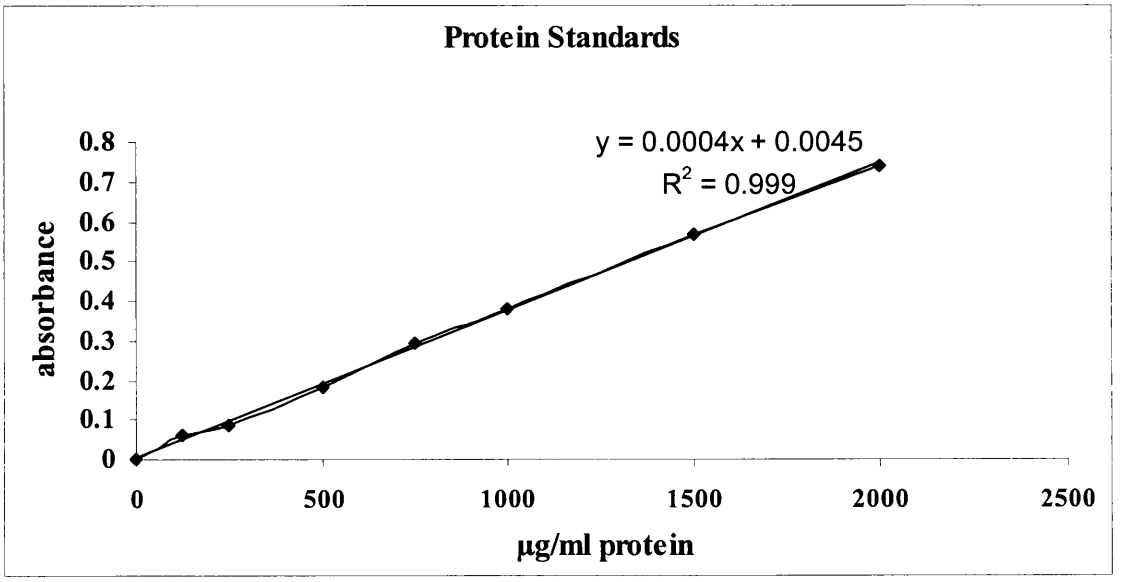


Figure 4.3. Protein standard graph from which unknown protein concentrations of samples were calculated.

4.3.2. SDS-PAGE

Protein profiles for *S. lacrymans* cultures exposed to *Trichoderma* VOCs are presented in figures 4.4a, 4.5a, 4.6a and 4.7a for *S. lacrymans* 12C, Forfar, H28 and BF050 respectively. Corresponding dendograms, which show the percentage similarity between the cultures exposed to *Trichoderma* VOCs and the respective *S. lacrymans* controls are shown in figures 4.4b – 4.7b.

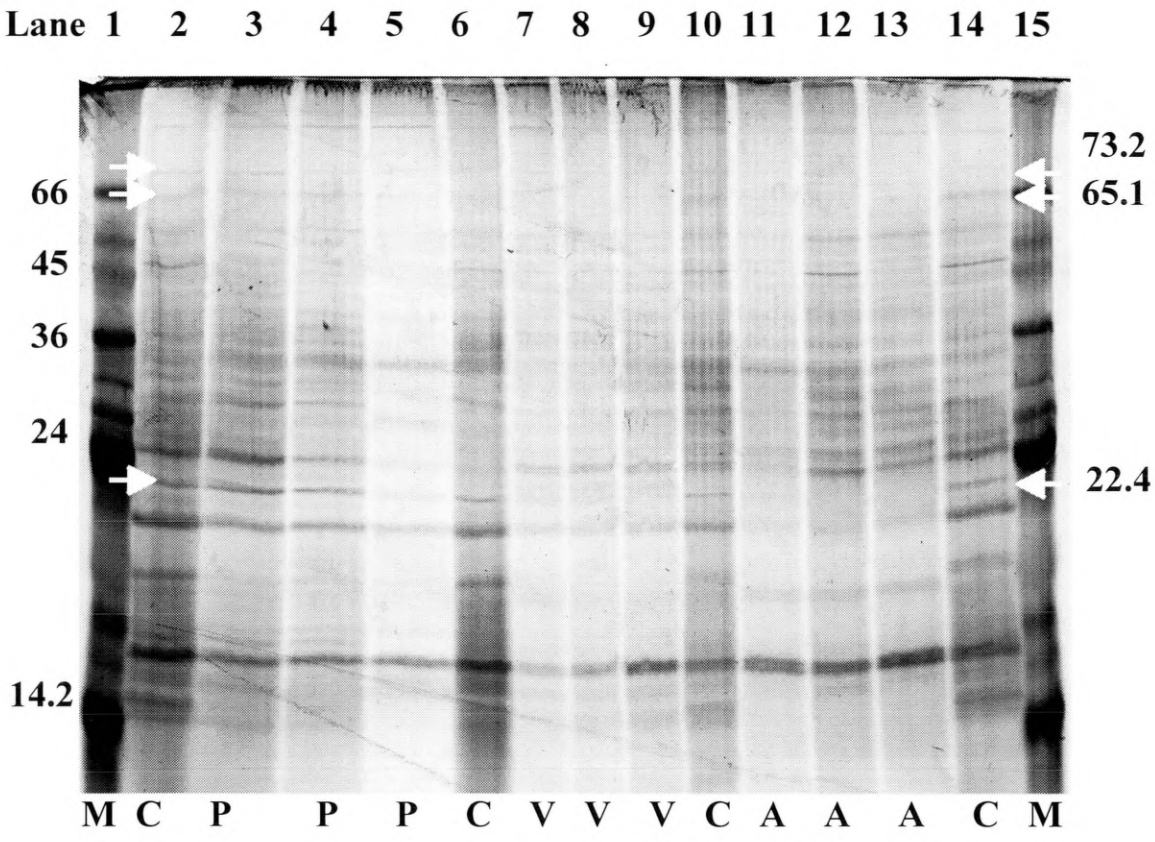


Figure 4.4a. Protein profile of *S. lacrymans* 12C; lanes 2,6,10 and 14 are the control culture which was not exposed to any antagonist; lanes 3-5 were grown in the presence of *T. pseudokoningii*; lanes 7-9 were grown in the presence of *T. viride* and lanes 11-13 were grown in the presence of *T. aureoviride*. Lanes 1 and 15 are the molecular weight markers. The MW of the proteins, in kilo-Daltons (kDa), affected by the *Trichoderma* VOCs are highlighted down the right hand side of the gel with weights of the markers in kDa on the left.

Although the majority of protein bands are reproduced in both controls as well as those exposed to VOCs there are a number of interesting differences. It is clear that the banding pattern for *T. pseudokoningii* is the same as that of the controls (Fig. 4.4a) and this is confirmed in the dendogram (Fig. 4.4b). Interestingly *T. pseudokoningii* had no inhibitory effect on the growth of *S. lacrymans* 12C (Fig. 4.8) and as can be seen from figure 4.4b the proteins produced by the control cultures and cultures of *S. lacrymans* 12C exposed to *T. pseudokoningii* were 100% similar. *T. aureoviride,* which had the greatest inhibitory effect (82%) on the growth of *S. lacrymans* 12C (Fig.4.8), also caused the greatest changes in the corresponding protein profiles. The production of proteins of molecular weights 22.4, 65.1, 73.2kDa were all inhibited by VOCs from *T. aureoviride* (Fig. 4.4a) and the protein profiles were only 86% similar to the control lanes (Fig. 4.4b). Growth inhibition of *S. lacrymans* 12C by *T. viride* was 41% (Fig. 4.8) and this corresponded with complete inhibition of the production of the protein with MW 65.1kDa. The synthesis of the 22.4 kDa protein however appears only to have been reduced by the VOCs from *T. viride* indicated by decreased band intensity shown in Fig.4.4a when compared with the control lanes. The protein profile from *T. viride* treatment was 94% similar to the control (Fig. 4.4b) and 90% similar to *T. aureoviride*, however this does not take into account the reduction in band intensity, it is only presence or absence of a band. The replicates of all samples were 100% similar to one another.

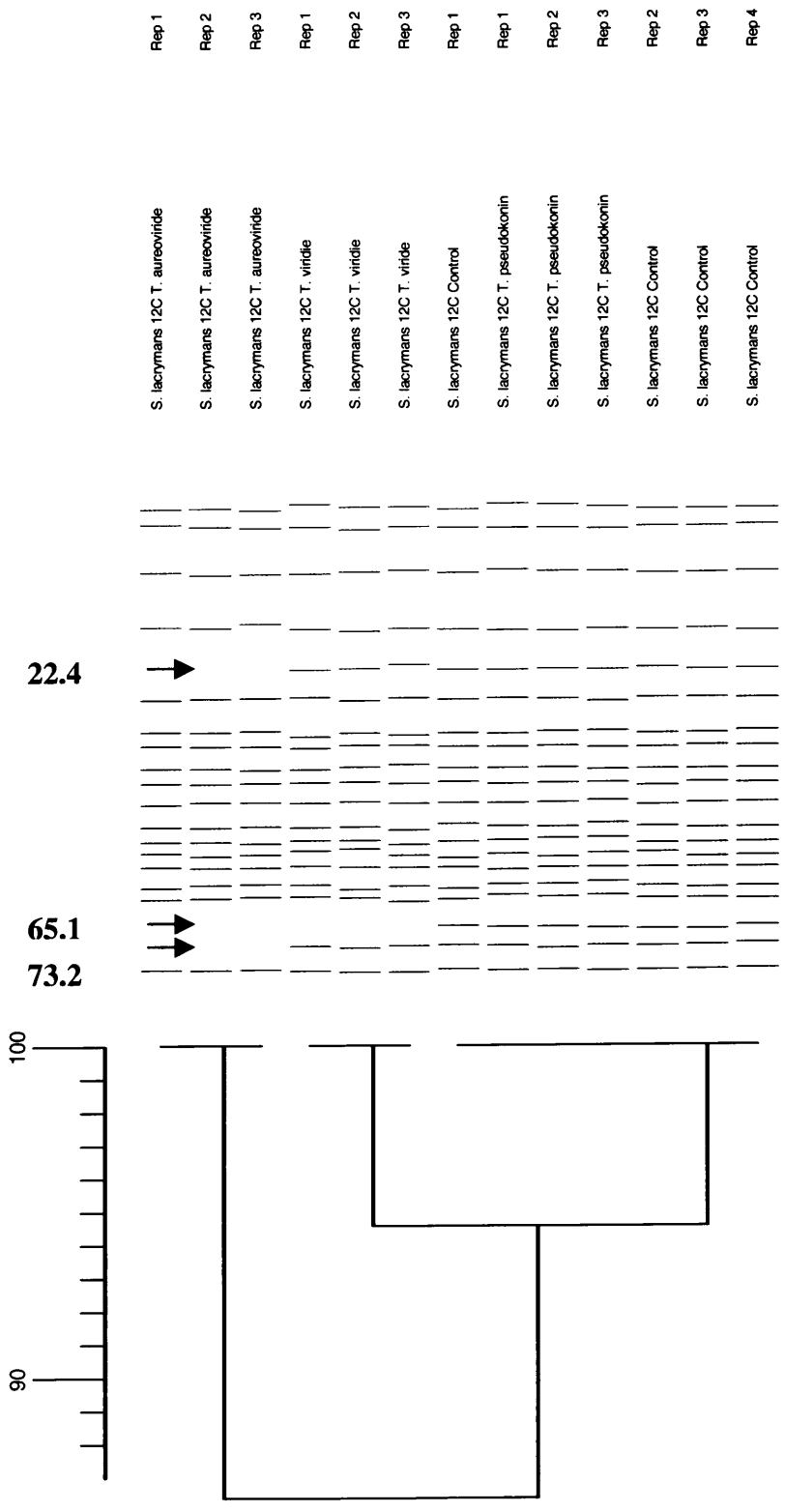


Figure 4.4b. Gelcompar generated dendogram of the bands produced by *S. lacrymans* 12C. The scale bar represents % similarity.

The protein profile for *S. lacrymans* Forfar is shown in Figure 4.5a with the corresponding dendogram in figure 4.5b. In this case, the same effect is clear for the protein band of 22.4 kDa size. VOCs from *T. aureoviride* have caused inhibition of the protein production, *T. viride* VOCs have reduced the extent of the protein synthesis and *T. pseudokoningii* VOCs had no effect (Fig. 4.5a). The VOCs have however had no effect on the protein of 65.1kDa size in this *Serpula* strain. A separate protein band (29.1 kDa) interestingly has been totally inhibited by the VOCs from both *T. aureoviride* and *T. viride*, but unaffected by *T. pseudokoningii* (Fig. 4.5a). Dendogram analysis in this case (Fig. 4.5b) show that *T. pseudokoningii* is 100% similar, *T. viride* 95% similar and *T. aureoviride* 92% similar when compared with the controls. *T. aureoviride* is 95% similar to *T. viride*. Again, this correlates well with percentage inhibition of growth by the antagonists (Fig. 4.8), which shows *T. pseudokoningii* inhibited *S. lacrymans* growth by 15%, *T. viride* by 54% and *T. aureoviride* by 92%.

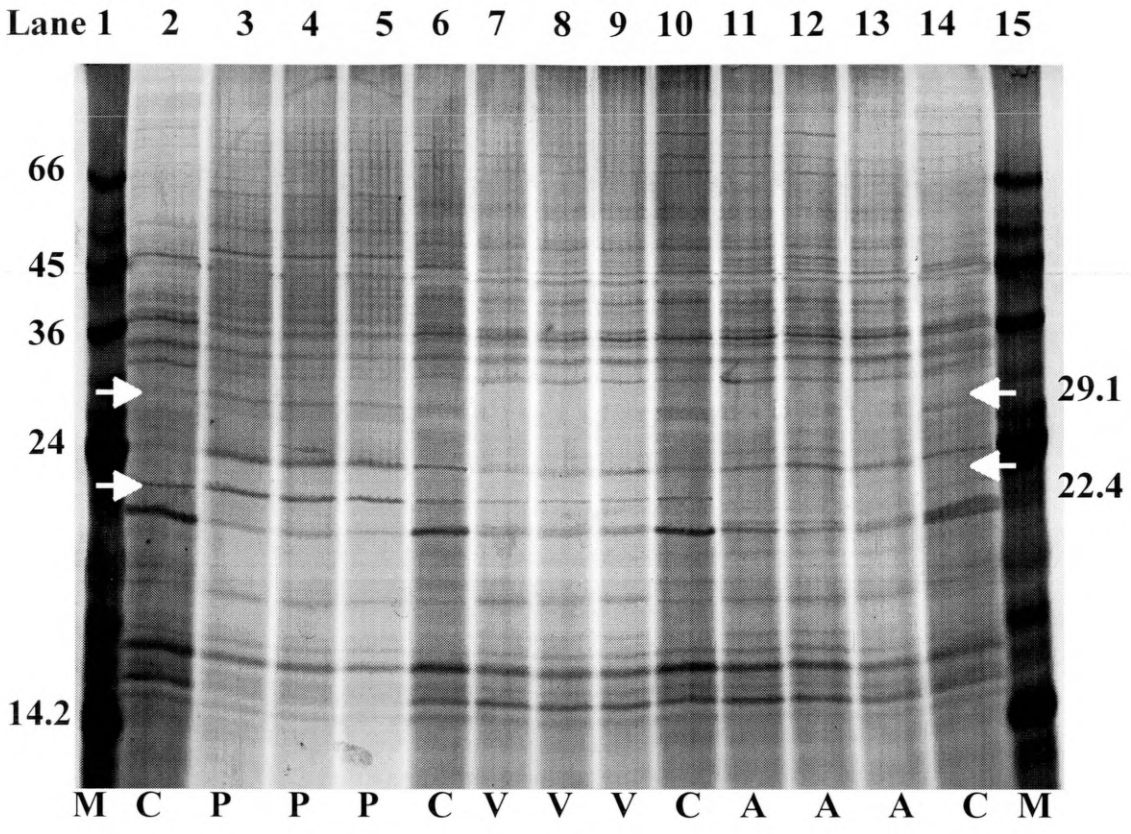


Figure 4.5a. Protein profile of *S. lacrymans* Forfar; lanes 2,6,10 and 14 are the control culture which was not exposed to any antagonist; lanes 3-5 were grown in the presence of *T. pseudokoningii*; lanes 7-9 were grown in the presence of *T. viride* and lanes 11-13 were grown in the presence of *T. aureoviride*. Lanes 1 and 15 are the molecular weight markers. The MW of the proteins in kDa affected by the *Trichoderma* VOCs are highlighted down the right hand side of the gel with weights of the markers in kDa on the left.

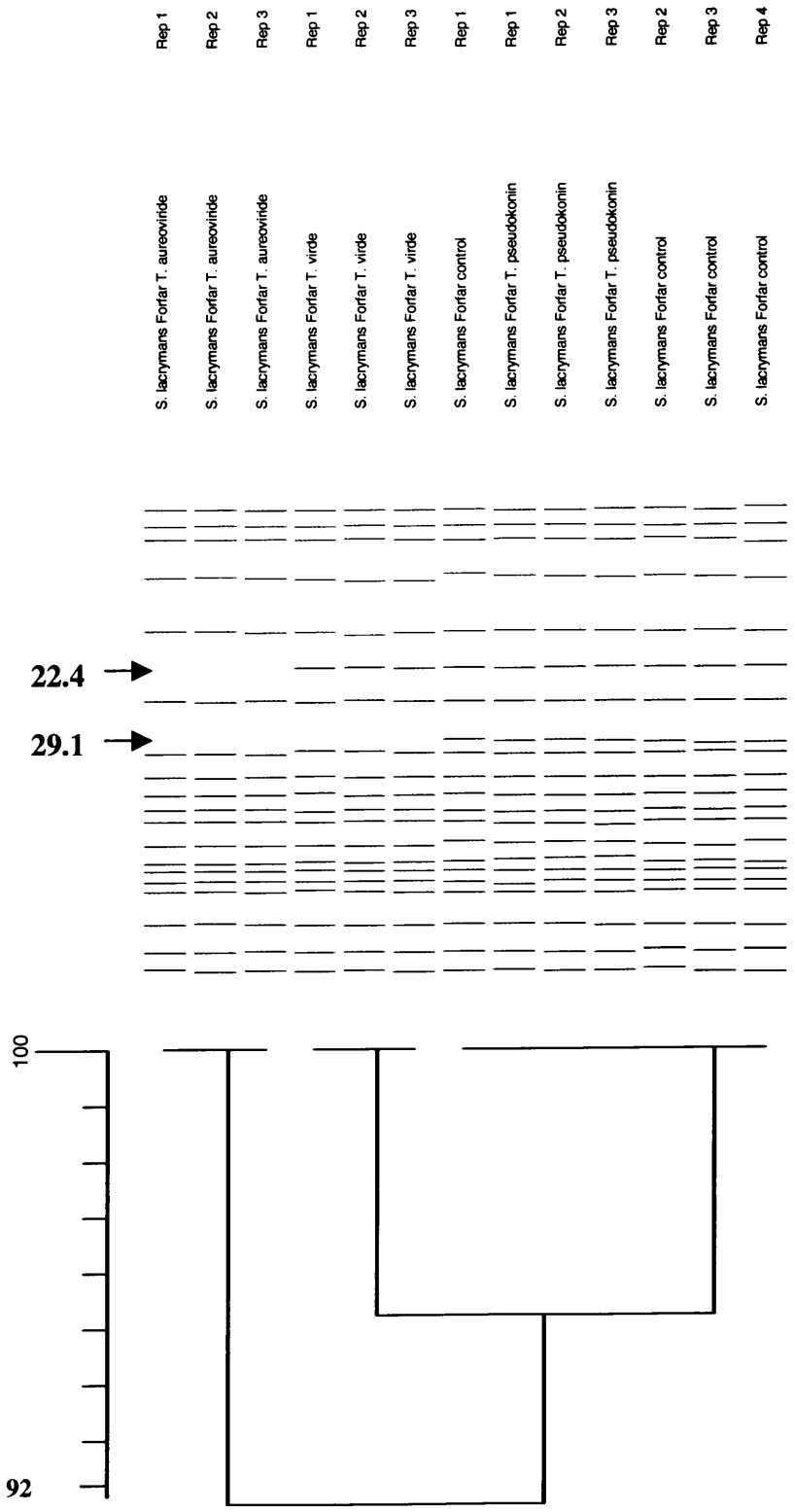


Figure 4.5b. Gelcompar generated dendogram of the bands produced by *S. lacrymans* Forfar. The scale bar represents % similarity.

The protein profile for *S. lacrymans* H28 is shown in figure 4.6a with the corresponding dendogram in figure 4.6b. The same effect is clear for the protein band at 22.4 kDa size as was seen for *S. lacrymans* 12C and Forfar, with VOCs from *T. aureoviride* completely inhibiting the protein production, *T. viride* having reduced the extent of the protein synthesis and *T. pseudokoningii* having no effect. The VOCs from both *T. aureoviride* and *T. viride* completely inhibited the synthesis of the protein band at 65.1 kDa size and *T. pseudokoningii* had no effect (Fig. 4.6a). The same effect on this protein was seen in *S. lacrymans* 12C (Fig. 4.4a), whereas there was no apparent effect on the protein 65.1 kDa in *S. lacrymans* Forfar (Fig. 4.5a). The synthesis of the protein at 29.1 kDa size was inhibited by VOCs from both *T. aureoviride* and *T. viride*, but unaffected by *T. pseudokoningii* VOCs (Fig. 4.6a). This is the same effect as seen for *S. lacrymans* Forfar (Fig. 4.5a). The VOCs had no effect on the protein of 73.2 kDa in this *Serpula* strain. Two different protein bands at 32.2 and 52.1 kDa, however have been totally inhibited by the VOCs from both *T. aureoviride* and *T. viride* but not *T. pseudokoningii* (Fig. 4.6a) in this *S. lacrymans* isolate. Dendogram analysis for *S. lacrymans* H28 (Fig. 4.6b) shows that *T. pseudokoningii* is 100% similar, *T. viride* and *T. aureoviride* are both 80% similar when compared with the controls however, *T. aureoviride* and *T. viride* are 94% similar to one another. Again, this correlates well with percentage inhibition of growth (Fig. 4.8), which shows *T. pseudokoningii* inhibited *S. lacrymans* growth by 2%, *T. viride* by 34% and *T. aureoviride* by 74%.

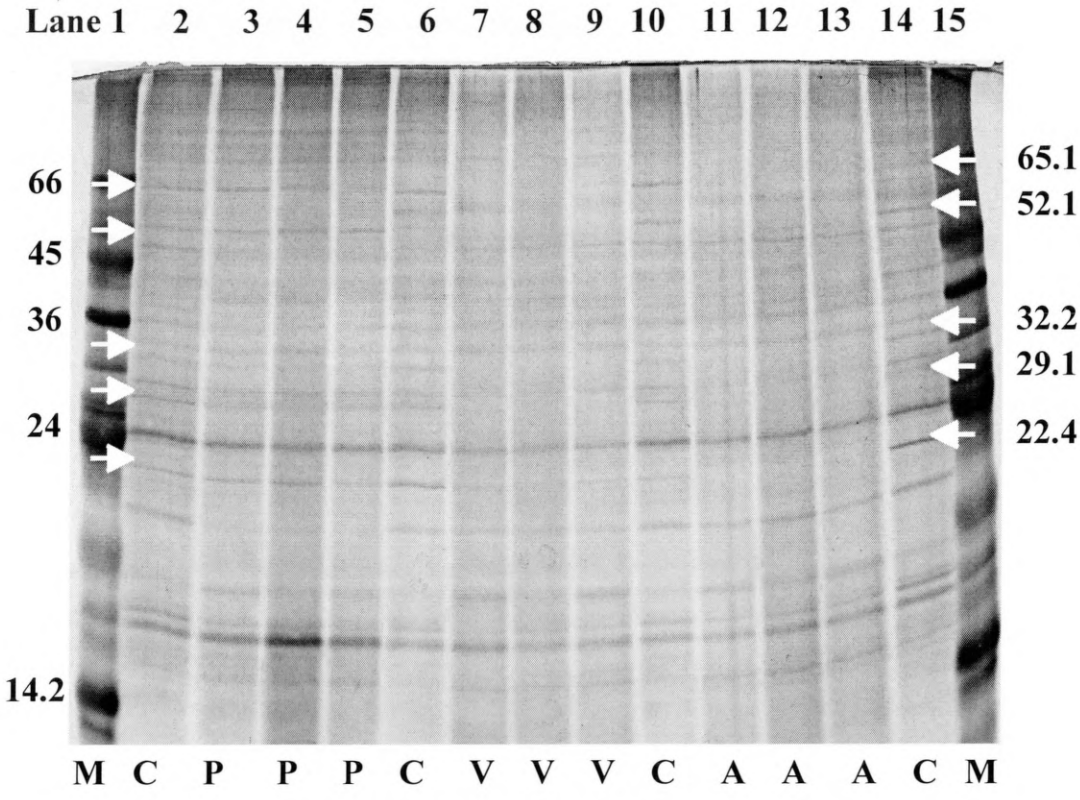


Figure 4.6a. Protein profile of *S. lacrymans* H28; lanes 2,6,10 and 14 are the control culture which was not exposed to any antagonist; lanes 3-5 were grown in the presence of *T. pseudokoningii*; lanes 7-9 were grown in the presence of *T. viride* and lanes 11-13 were grown in the presence of *T. aureoviride*. Lanes 1 and 15 are molecular weight markers. The proteins affected by the *Trichoderma* VOCs can be seen down the right hand side of the gel with weights of the markers in kDa on the left.

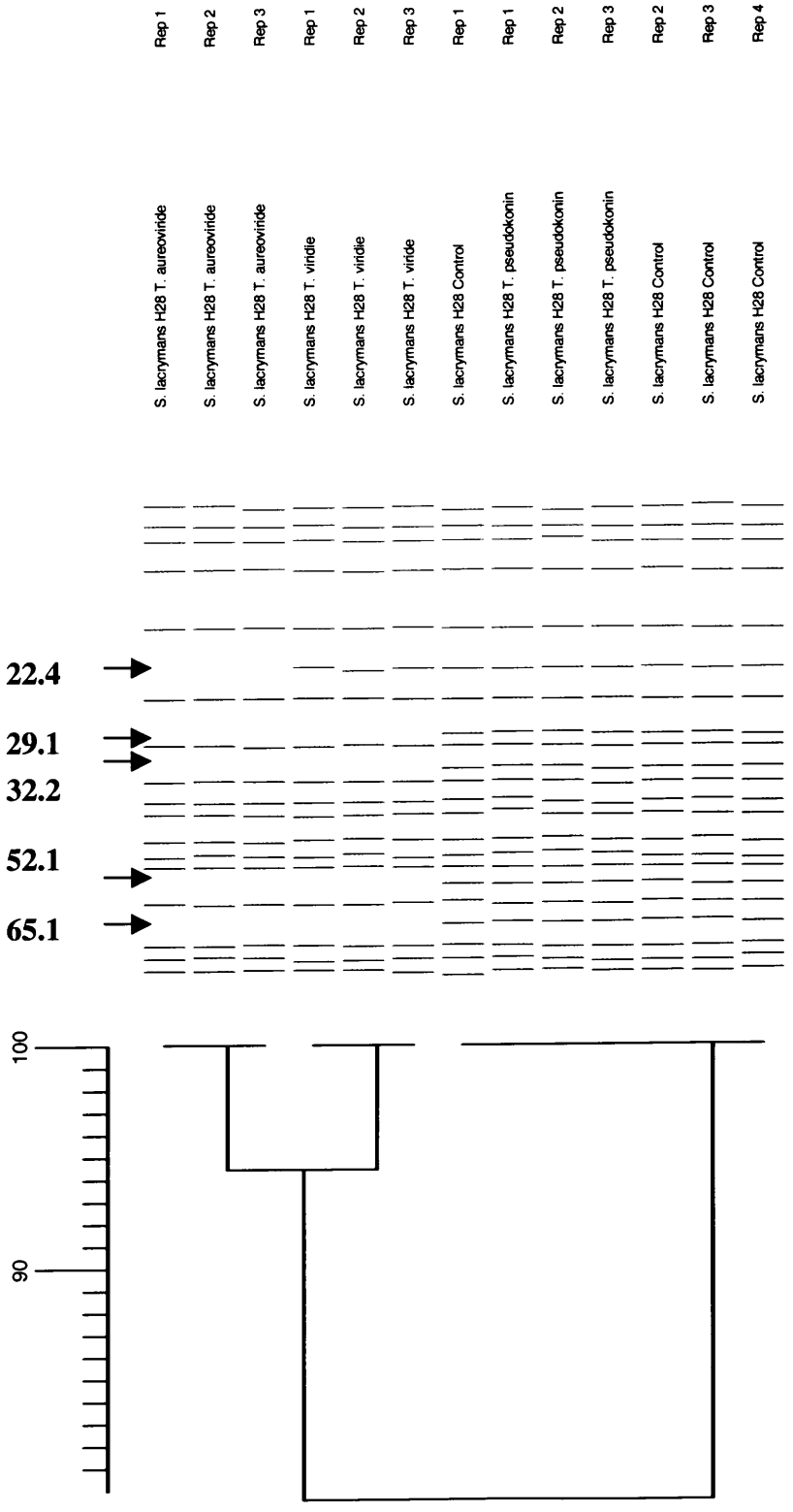


Figure 4.6b. Gelcompar generated dendogram of the bands produced by *S. lacrymans* H28. The scale bar represents % similarity.

The protein profile for *S. lacrymans* BF050 is shown in figure 4.7a with the corresponding dendogram in figure 4.7b. Once again, the effect on the protein band at 22.4 kDa is identical to that in *S. lacrymans* 12C, Forfar and H28. VOCs from *T. aureoviride* inhibited the production of the protein with MW 73.2 kDa whereas *T. viride* and *T. pseudokoningii* had no apparent effect on this protein (Fig. 4.7a). This mirrors the effect seen in *S. lacrymans* 12C (Fig. 4.4a), however there was no effect on this protein for *S. lacrymans* Forfar (Fig. 4.5a) and H28 (Fig. 4.6a). Surprisingly, the VOCs from all *Trichoderma* isolates had no effect on the proteins of 29.1 and 32.2 kDa and this *Serpula* strain does not have proteins of MW 52.1 and 65.1kDa. A separate protein band at 67.5 kDa however has been totally inhibited by the VOCs from both *T. aureoviride* and *T. viride* but not *T. pseudokoningii* (Fig. 4.7a). Dendogram analysis for *S. lacrymans* BF050 (Fig. 4.6b) shows that *T. pseudokoningii* is 100% similar, *T. viride* 93% similar and *T. aureovirde* 83% similar when compared with the controls. *T. viride* is 87% similar to *T. aureoviride*. As with the other 3 *S. lacrymans* isolates this correlates well with percentage inhibition of growth (Fig. 4.8), which shows *T. pseudokoningii* inhibited *S. lacrymans* growth by 10%, *T. viride* by 50% and *T. aureoviride* by 80%.

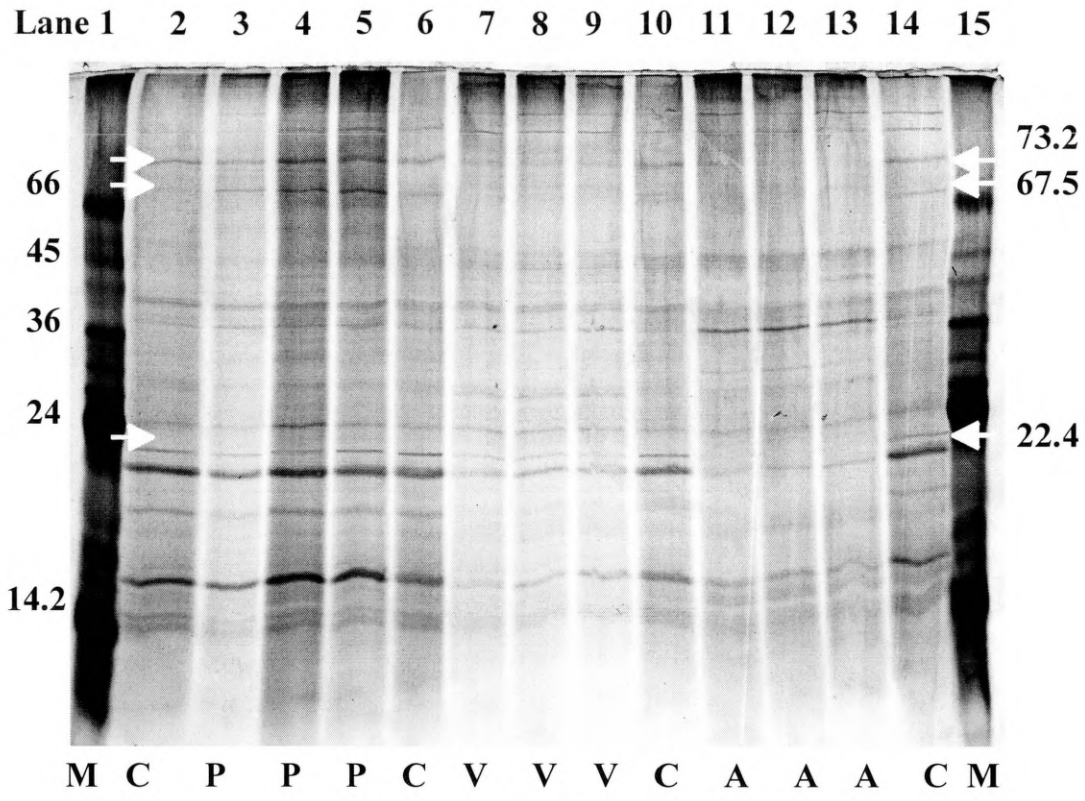


Figure 4.7a. Protein profile of *S. lacrymans* BF050; lanes 2,6,10 and 14 are the control culture which was not exposed to any antagonist; lanes 3-5 were grown in the presence of *T. pseudokoningii*; lanes 7-9 were grown in the presence of *T. viride* and lanes 11-13 were grown in the presence of *T. aureoviride*. Lanes 1 and 15 are molecular weight markers. The proteins affected by the *Trichoderma* VOCs can be seen down the right hand side of the gel with weights of the markers in kDa on the left.

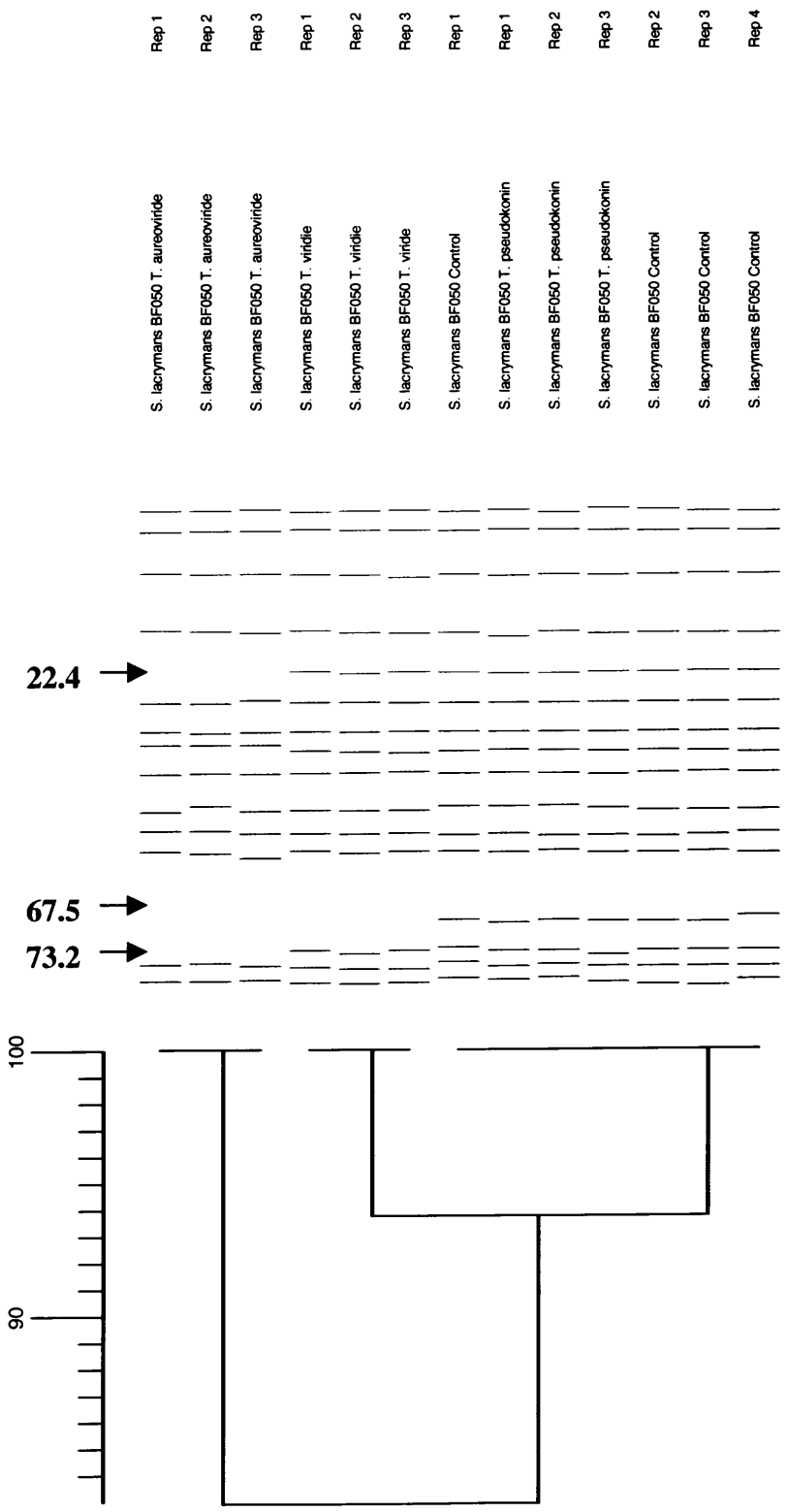


Figure 4.7b. Gelcompar generated dendogram of the bands produced by *S. lacrymans* BF050. The scale bar represents % similarity.

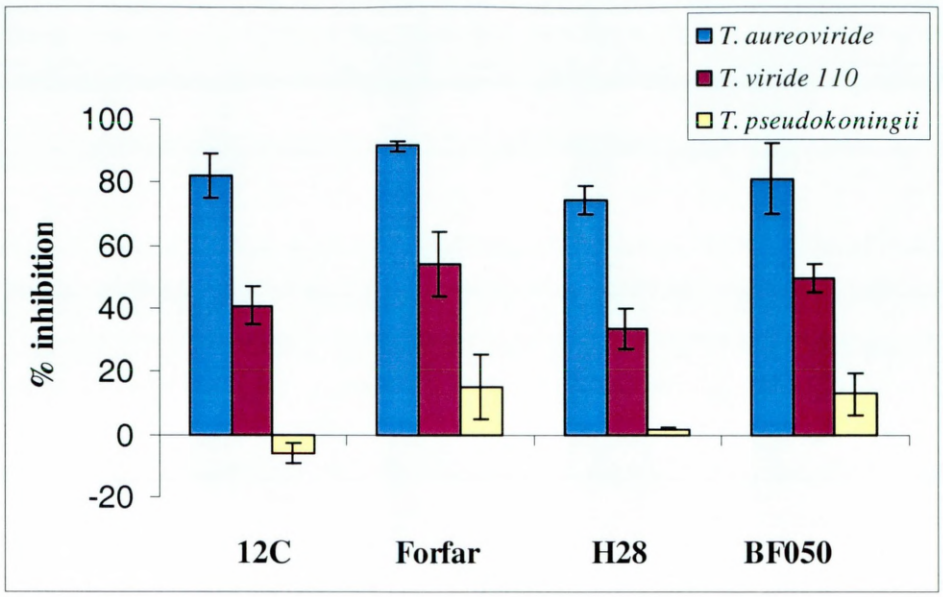


Figure 4.8. Growth inhibition (%) of four *S. lacrymans* isolates exposed to the VOCs from the three *Trichoderma* isolates (Results from Chapter 2 section 2.2.2). Bars represent standard deviations.

Comparisons of the protein profiles in each of the *S. lacrymans* controls compared with one another can be seen in figure 4.9. This shows that the protein profiles for the 4 *S. lacrymans* strains all differ slightly from one another. The results clearly show that of the 4 strains, BF050 exhibits the most differences from the other 3 isolates. *S. lacrymans* 12C, Forfar and H28 are very similar to one another (always about 80%), whereas BF050 is never more that 68% similar to any of the strains. The protein profiles have many bands in common and a basic banding pattern can be recognized despite the differences. Protein bands at 15.5, 16.1, 17.6, 20.2, 22.4, 24.6, 27.5, 29.1, 32.2, 35.5, 38.5, 44.9 73.2 and 85 kDa size are common to all 4 isolates. Protein bands at 33.1, 41.9, 47.7, 52.1, 55.2 and 65.1kDa are common to *S. lacrymans* 12C, Forfar and H28.

Isolate	Forfar	H28	BF050
12C	83%	80%	65%
Forfar	-	82%	58%
H28	-	-	68%

Table 4.1. Comparison (percentage similarity) in protein profiles of each *S. lacrymans* strain compared with one another.

It is clear from the results that all 4 S. *lacrymans* isolates showed differing sensitivities to the *Trichoderma* VOCs. Overall, *S. lacrymans* H28 demonstrated the greatest number of changes in protein expression and *S. lacrymans* Forfar showed the least when exposed to the Trichoderma VOCs. *T. aureoviride* and *T. viride* totally inhibited the production of the protein at 65.1kDa but only in *S. lacrymans* 12C and H28. The protein of molecular weight 73.2kDa was completely inhibited by *T. aureoviride* for *S. lacrymans* 12C and by both *T. aureoviride* and *T. viride* for *S. lacrymans* BF050. The most consistently observed effect was directed against the 22.4 kDa band. The VOCs from *T. aureoviride* completely inhibited the synthesis of this protein in all *Serpula* isolates, while VOCs from *T. viride* caused a marked reduction in synthesis of the 22.4kDa, which was again consistent for all *Serpula* isolates. VOCs from *T. pseudokoningii* had no effect on the protein in any strain. The repetition of the experiment resulted in gels for which there was slight variation in definition and intensity of proteins within lanes, especially proteins >66kDa to those presented here. The overall banding pattern however and subsequent analysis was identical to the results presented and discussed for the first experiment (see Appendix B, 2.1), demonstrating the reproducibility and robustness of the test system.

4.4. Discussion

SDS-PAGE combined with silver staining is a valuable tool used widely in mycology to analyse proteins found in fungal extracts. Wood decay basidiomycetes such as *H. annosum* (Palfreyman *et al.*, 1990), *S. lacrymans* (Palfreyman *et al.*, 1991) and *L. lepideus* (Glancy, 1990) have been analysed using SDS-PAGE combined with silver staining. Comparisons of banding patterns can give information on the molecular weight of specific proteins and show similarities and differences between extracts. SDS-PAGE combined with silver staining was used in this project to try and identify any changes in protein profiles of *S. lacrymans* isolates when exposed to VOCs from *Trichoderma* isolates which give varying inhibitory effects on growth.

The results presented show that production of some fungal proteins is inhibited in the presence of inhibitory *Trichoderma* VOCs and banding patterns achieved were reproducible across replicates and when the plate experiment and subsequent analysis was repeated. The results show that after 7 days of interaction, protein synthesis by all *S. lacrymans* isolates was affected by the volatile secondary metabolites of *T. aureoviride and T. viride,* but not by those of *T. pseudokoningii.* The response of the *S. lacrymans* isolates is strain specific to both the *Serpula* isolate and the *Trichoderma* strain, with *S. lacrymans* H28 showing the greatest change in protein expression and *S. lacrymans* Forfar showing the least change. Despite the fact that this later strain showed greatest growth inhibition when grown on malt extract medium and H28 showed the least growth inhibition (Fig. 4.8). However, the fact that H28 is less sensitive might imply that there would be a need for greater changes at the molecular level for this strain to overcome such VOC stresses.

The protein profile of *S. lacrymans* BF050 is significantly different from the other 3

S. lacrymans isolates. Vigrow (1992) compared 14 *S. lacrymans* building isolates from around the world, grown in malt extract broth with the standard strain FPRL 12C and found that the over-riding visual impression was that only 2 isolates, BF050 and BF015B, differed noticeably from *S. lacrymans* 12C. Vigrow *et al.* (1991b) then used western blotting to further identify the 2 atypical *Serpula* isolates and discovered that BF015B was in fact *S. himantiodes* and BF050 was an isolate of *S. lacrymans*. It is interesting that the protein profiles of the 2 building isolates (BF050 and Forfar) are the least similar to one another than any other combination and that the 'wild' Himalayan isolate is so similar to the *S. lacrymans* Forfar building isolate. White *et al.* (2001) analysed genetic fingerprints of *S. lacrymans* and *S. himantiodes* isolates of wild Himalayan woodland and building origin using RAPD-primer combinations. Included in this study were *S. lacrymans* 12C, Forfar, H28 and BF050. The *S. lacrymans* isolates were never 100% similar to each other but *S. lacrymans* 12C, Forfar and H28 were more similar to one another (around 80% similar) than BF050 (around 70% similar). The results demonstrated that *S. lacrymans* and *S. himantiodes* were clearly distinguished but the banding patterns of the Himalayan *S. lacrymans* isolates were not distinct from those of building isolates. RAPD-PCR has previously successfully distinguished between subgroups of fungi (Cooke *et al.*, 1996), but White *et al.*, (2001) found there was no association between the RAPD based DNA fingerprints and geographical origin of the isolates.

While there were differences in protein bands affected by the *Trichoderma* VOCs for each of the *S. lacrymans* strains, the protein at MW 22.4 kDa showed the same response in all 4 of the *Serpula* isolates. Production of this protein appeared to correlate well with growth inhibition as production of this protein was completely inhibited by the VOCs from *T. aureoviride*, which gave the greatest levels of inhibition of the *S.*

lacrymans isolates. The protein was also unaffected by VOCs from *T. pseudokoningii*, which gave very little or no inhibition of growth of *S. lacrymans* and the VOCs from *T. viride* (which gave on average between 35-50% inhibition of growth) caused a marked reduction in synthesis of the 22.4kDa protein. It is possible that this protein is associated with mycelial development in *S. lacrymans*, as the degree of mycelial growth inhibition was related to the reduction in synthesis of this protein. Excising this band, digesting it and identifying the protein using electrospray ionization mass spectrometry or matrix-assisted laser-desorption ionization time of flight, which would measure the masses of the peptides making up the protein, could allow characterization of the protein. Databases could then be searched to try and find a match for the sequence of masses, which would allow identification of the protein perhaps indicating if it is a critical component in mycelial development.

While none of the strains showed 100% similarity, protein bands at 15.5, 16.1, 17.6, 20.2, 22.4, 24.6, 27.5, 29.1, 32.2, 35.5, 38.5, 44.9 73.2 and 85 kDa size are common to all 4 isolates. Protein bands at 33.1, 41.9, 47.7, 52.1, 55.2 and 65.1kDa are common to *S. lacrymans* 12C, Forfar and H28 and a basic banding pattern could be recognized. Of these common proteins, 14 of them were also common to all building isolates tested by Vigrow (1992), including the protein band at 22.4kDa. Although the protein bands produced in this experiment do not match all the bands Vigrow (1992) found, there were differences in the experimental procedure. Vigrow (1992) grew the *S. lacrymans* isolates on malt extract broth not agar, the gel was a 5-15% gradient gel not a 15% single concentration gel and the extraction procedure varied slightly. Vigrow *et al.* (1989) reported that it is important to compare cultures grown under conditions as identical as possible and this may in part explain some of the differences seen.

Altered levels of protein synthesis are directly linked to enhanced expression or repression of particular genes, therefore VOCs may be influencing the gene expression of the organism. Changes in the growth and extracellular enzyme activity were demonstrated to be a direct influence of the VOCs (Chapter 2). This included significant levels of inhibition of the *S. lacrymans* isolates by a number of the *Trichoderma* isolates and increased peroxidase production being linked to increased pigmentation associated with a stress reaction in the fungus. Although protein bands have not been fully characterized at this point, some of the changes seen to protein profiles may explain the major changes in growth and extracellular enzyme activity. This may include increases or decreases in protein synthesis, which was not examined in this study, except for protein at MW 22.4 kDa. No extra bands, however, appeared in the protein profiles of *S. lacrymans* isolates exposed to *Trichoderma* VOCs when compared with control samples. Therefore, it is unlikely that there is any link to production of stress metabolites, as these proteins would have resulted in the appearance of extra bands. For example, heat stress in *S. lacrymans* results in the production of heat shock proteins (Sienkiewicz, 1999). It would be interesting to expose the *S. lacrymans* isolates to the VOCs identified in chapter 3 as possible inhibitory compounds or other environmental stresses to determine if these compounds or other stresses also caused the repression of the protein at molecular weight 22.4kDa or any of the other proteins affected by the *Trichoderma* VOCs.

While the combination of SDS-PAGE and silver staining is a highly sensitive technique allowing the detection of nanogram amounts of protein, only the soluble proteins produced in a mycelial culture have been assessed in this study. Other techniques such as 2-Dimensional SDS-PAGE or Isoelectric focusing, which would show a greater variety of proteins, or using specific molecular probes may produce information relating

biological properties to specific molecular components. The fact that the effect of the VOCs was fungistatic rather than fungicidal may mean their effect on the production of proteins is subtle and may include increases or decreases in protein production rather than synthesis simply being switched on or off.

CHAPTER 5: Protein profile modulation and identification of target proteins after exposure to *Trichoderma* VOCs

5.1 Introduction

When fungi and bacteria are exposed to elevated temperatures, they respond by synthesizing a small number of highly conserved proteins known as the heat-shock proteins or stress proteins (Sienkiewicz, 1999). The production of heat shock proteins has also been reported in response to other environmental stresses, such as heavy metals (Sanchez *et al.*, 1992) and hydrogen peroxide (Collinson and Dawes, 1992). It has been recognized that after this stress is removed normal protein production gradually resumes (DiDomenico *et al.*, 1982; Plesofsky-Vig and Brambl, 1985). The results from chapter 4 determined that even though there didn't appear to be production of any identifiable stress proteins, *Trichoderma* VOCs inhibited the production of individual proteins in *S. lacrymans*. It was not established however whether production of these proteins would return to normal after removal of the volatile stress.

Melin *et al.* (2002) analysed the effect of an antibiotic produced by *Streptomyces* on protein levels in the filamentous fungus *Aspergillus nidulans*. Two dimensional gel electrophoresis demonstrated that the production of 20 proteins was influenced by exposure to the antibiotic, with production of these proteins either increasing or decreasing. Five of the proteins were identified using peptide analysis by mass spectrometry, of these, one of the down-regulated proteins was identified as glyceraldehyde dehydrogenase, a protein involved in general metabolic pathways. None of the proteins highlighted in chapter 4 have been characterized, but of all the proteins inhibited, the protein at 22.4 kDa appears to be most likely linked to a metabolic pathway in *S. lacrymans*. It is possible that this protein is associated with mycelial development in *S. lacrymans*, as the degree of mycelial growth inhibition was related to the reduction in synthesis of this protein. Identification of an individual protein can be achieved by a range of analytical methods that examine the peptide

framework of the compound. Specific cleavage of the polypeptide backbone of a protein by proteolytic enzymes or chemical agents results in a set of peptides, the peptide map, which covers the entire sequence of the protein. The peptide map obtained by specific enzymatic or chemical cleavage is a unique fingerprint for a given protein. Mass information of peptides can be easily obtained by Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) or Electrospray ionization mass spectrometry (ESI-MS). A mass-spectrometric peptide map contains the experimentally determined molecular weights of the derived peptides. The experimental data is a partial or complete set of molecular weights of peptides resulting from a given cleavage of the protein. Searching a database with the molecular weights of the peptides will result in a list of matching proteins usually ordered by decreasing number of peptide matches (Mann *et al.* 1993).

The aim of this chapter was to determine whether *Trichoderma* VOCs affected protein regulation in *S. lacrymans*. This will be achieved by exposing selected *S. lacrymans* isolates to VOCs from an antagonistic *Trichoderma* species, removing the stress and comparing protein profiles produced under stressed conditions against those of mycelia grown after the stress has been removed. Thereby determining if protein production had returned to normal. Finally, ESI-MS will be used to try to identify proteins of interest.

5.2. Materials and methods

5.2.1. VOC mediated interactions

Identical tests were set up as described in Chapter 2 section 2.2.2 except that only one *Trichoderma* isolate was used, *T. aureoviride* 91968, which was grown on 3% malt extract (Oxoid, Hampshire, UK). This strain was selected on the basis that it had the greatest influence on protein production of *S. lacrymans* and completely inhibited the protein at 22.4 kDa. As previously (Chapter 2, section 2.2.1), four *S. lacrymans* isolates were used, which were grown on 5% malt extract/ 2% agar. Controls were set up for each *S. lacrymans* isolate and they contained no *Trichoderma* inocula on the bottom plate. Six replicates were set up for all tests and controls. All cultures were incubated at 21°C until the growth of the *S. lacrymans* in the controls had nearly reached the edge of the plate (usually 7 days). After the 7 days incubation at 21°C, the over and under plates were separated and plates containing the *Trichoderma* were discarded. At this time, the perimeter of the *S. lacrymans* growth was marked on the underside of the Petri dishes. The *S. lacrymans* plates were then covered with sterile Petri dish lids and returned to the incubator for another 5 days at 21°C. Parallel control plates, in which the *S. lacrymans* isolates were grown on the same media, but without the *Trichoderma* spp. antagonists, were incubated for the duration alongside the plates that were being challenged with the antagonists.

5.2.2 Extraction of proteins

Gloves were worn at all times and all procedures were carried out on ice.

After the 12 days incubation at 21°C, the mycelium was separated into areas that had grown under the influence of the antagonist and areas of re-growth after removal of the antagonist (Fig. 5.1). The original inoculation cores were removed and the remaining

areas of *S. lacrymans* mycelia were harvested separately using a spatula to gently remove the mycelia from the agar. Two plates of each treatment were pooled resulting in 3 replicates for each treatment and control. All mycelia were washed 3 times in phosphate buffered saline (PBS) containing proteinase inhibitors (see Appendix B, 1.1), then frozen in liquid nitrogen and lyophilised overnight (Edwards, UK).

The lyophilised mycelia were ground in liquid nitrogen in a pre-chilled mortar and pestle, using acid purified sand (Fluka, Dorset, UK) as the grinding agent. Once a fine powder was achieved, it was transferred to a 1.5ml Eppendorf and 600µl of ice cold PBS containing the proteinase inhibitors was added. The sample was homogenized by vortexing for 30sec and grinding using a micro-pestle. Samples were spun at 13, 200 rpm for 10 minutes at 4°C in a refrigerated microcentrifuge (Sigma Laboratories, Germany) and the resulting pellet was discarded and the supernatant retained.

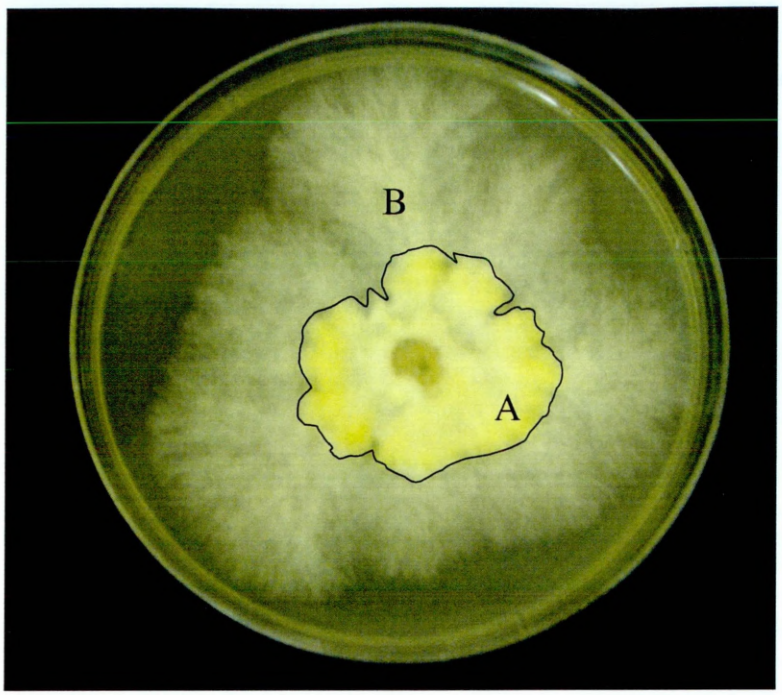


Fig. 5.1. Picture of *S. lacrymans* growth showing part of colony exposed to *Trichoderma* VOCs (A) and new growing edge of *S. lacrymans* colony after removal of the antagonistic stress (B). Line represents area marked on underside of plate to separate areas of growth under stressed and non-stressed conditions.

5.2.3. Protein concentration

The concentration of protein present in the supernatant was determined following the method in chapter 4, section 4.2.3.

5.2.4. Preparation of acrylamide gels

Gels were prepared as described in Chapter 4, section 4.2.4.

5.2.5. Sample preparation

The diluted aliquots of *S. lacrymans* isolate were thawed on ice, then mixed in a 1:1 ratio with Laemmli sample buffer (Appendix B, 1.5), boiled for 5 minutes and microcentrifuged at 13, 200rpm for 5 minutes. Forty μl of each sample was loaded into the wells of the gel ensuring that replicate samples were loaded side by side and

interspaced with equal lanes of the *S. lacrymans* control. On each gel, there were 9 lanes of samples: 3 replicate lanes of *S. lacrymans* exposed to *T. aureoviride*; 3 replicate lanes of *S. lacrymans* grown after removal of the antagonist and 3 replicate lanes of control samples. The final 2 lanes at either side of the gel contained molecular weight markers as described in chapter 4, section 4.2.6.

5.2.6. Electrophoresis and silver staining

Electrophoresis of the gels was carried out following the methods in chapter 4, section 4.2.7 and the gels were stained using the Bio-rad silver stain plus kit catalogue No. 161-0449 (Bio-rad, Hertfordshire, UK) following the method described in chapter 4, section 4.2.8.

5.2.7. Data analysis

Gel images were scanned using an Epson scanner 1640XL (Getech, Suffolk, UK) and the images processed using GelCompar (Applied Maths, Kortrijk, Belgium), as described in chapter 4, section 4.2.9.

5.2.8. Protein identification

Two of the original stock replicates of *S. lacrymans* H28 exposed to *T. aureoviride* and 2 of the controls (Chapter 4, section 4.2.2) were thawed on ice and 2.5 × volume of ice-cold acetone was added. The samples were returned to the freezer and left overnight. The samples were then spun at 13, 200 rpm for 10 minutes at 4°C in a refrigerated microcentrifuge (Sigma Laboratories, Germany) and the acetone pipetted off and the pellet retained. Fifty µl of Laemmli sample buffer (Appendix B, 1.5), was added to the control samples and 25 µl was added to the samples exposed to the *T. aureoviride*

VOCs. Double the amount of sample buffer was added to the control as it had a greater concentration of protein than the sample exposed to *T. aureoviride* VOCs, so while it was not vital to have exactly equal concentrations of protein in the control and test sample, by adding double the amount of sample buffer to the control this diluted the sample to make it more comparable with the test sample, which had a much lower protein concentration. Both the control samples and the test sample were loaded on the gel, as the band to be excised in the control sample will be missing in the test sample. Therefore, this ensures the correct band will be excised and also shows that this band is definitely inhibited in the test sample. The samples were then boiled for 5 minutes and microcentrifuged at 13, 200 rpm for 5 minutes. Twenty µl of each sample was loaded into the wells of a Tris-HCl 15% resolving gel, 4% stacking gel, 10 well Ready gel (Bio-rad, Hertfordshire, UK) and run using a Mini-PROTEAN II Cell and an electrophoresis powerpack unit (Bio-rad laboratories, Hertfordshire, UK), at 40mA for 2 hours or until the dye front had reached the end of the gel. Directly after electrophoresis the two plates were separated carefully using a palette knife and the gel was Coomassie stained.

5.2.8.1. Coomassie staining

The gel was placed in a large sterile Petri dish (14.5cm diameter) and submerged in Coomassie staining solution (Chen *et al.*, 1993):

Methanol	25ml
Glacial acetic acid	5ml
u-p dH_2O	20ml
Coomassie brilliant blue R-250 (Sigma)	0.1g

The gel was left to soak in the Coomassie staining solution on a rocking platform for 2 hours. After this time the Coomassie solution was discarded and the gel was covered with destaining solution:

Methanol	125ml
Glacial acetic acid	25ml
u-p dH_2O	100ml

The destain solution was replaced with fresh solutions several times before being left in the solution overnight until the background of the gel was clear.

5.2.8.2. In-gel digest procedure

The gel was placed onto a glass plate that had been soaked in 10% Decon® (Decon Laboratories, Sussex, UK) overnight, washed with hot water, rinsed in up-dH_2O and wiped with 100% ethanol. The band at 22.4 kDa was excised from the 2 control lanes using a sterile scalpel blade. The bands were diced into small pieces ($1mm^2$) and placed into a 0.5 ml Eppendorf tube. One hundred µl of 25 mM ammonium bicarbonate (NH_4HCO_3) in 50% Acetonitrile (ACN) was added to the gel pieces, vortexed for 10 min and then pipetted off. This step was repeated twice.

5.2.8.3. Reduction and alkylation

The gel pieces were dried for 20 minutes in a Speed-vac™ (Savant, UK). Twenty five µl of 10 µM DTT in 25 mM NH_4HCO_3 was added to the dried gel pieces and microcentrifuged at 13, 200rpm for 2 minutes. The tubes were then placed in a heating block at 56°C for 1 hour. After the hour the solution was removed and 25µl of 5 mM iodoacetamide in 25 mM NH_4HCO_3 was added, vortexed and microcentrifuged at

13, 200rpm for 2 minutes. The tubes were wrapped in tin foil and left in the dark at room temperature for 45 minutes. After this time the solution was removed and the gel pieces washed with 100µl of NH_4HCO_3, vortexed for 10 minutes and microcentrifuged at 13, 200rpm for 2 minutes. The solution was then removed and the gel pieces were dehydrated with 100µl of 25 mM NH_4HCO_3 in 50% ACN, vortexed for 5 minutes, microcentrifuged at 13, 200rpm for 2 minutes and repeated once. The gel pieces were dried for 20 minutes in a speed-vac prior to trypsin digest.

5.2.8.4. Trypsin digest

The dried gel volume was estimated in µl and 3 × volume of 12.5 ng/µl trypsin in 25mM NH_4HCO_3 (freshly diluted) was added and vortexed for 10 minutes. The gel pieces were then incubated at 4°C for 30 minutes. If after this time any trypsin solution was left it was pipetted off, then enough 25 mM NH_4HCO_3 was added to cover the gel pieces. The samples were microcentrifuged at 13, 200rpm for 2 minutes and the tubes covered in Nescofilm and incubated at 37°C overnight (at least 16 hours). After this time the gel pieces were then completely dried for 30 minutes in a Speed-vac™.

5.2.9. Electrospray ionisation mass spectrometry

The 2 replicate samples from the control lanes along with a known protein (Alpha haemoglobin) were run on a ThermoFinnigan LCQ-DECA Ion trap mass spectrometer (San Jose, CA, USA) after digestion with trypsin. The known protein acts as an internal standard as the database search of the peptide masses from this protein should result in a match for Alpha haemoglobin. Twenty µl of a 50:50:1 (v: v: v) ratio of methanol, water and acetic acid was added to the samples.

A 5µl injection from the samples was made onto a reversed phase column, LUNA C18 (150mm x 1mm). Solvent A was 0.4% acetic acid, 0.01% heptafluorobutyric acid (HFBA) in water, solvent B was 0.01% HFBA in 70% acetonitrile. Peptides were eluted from the column with a linear gradient of 100-0% of solvent A and a linear gradient of 0-100% solvent B at a flow rate of 50µl/min for 40 minutes. The column eluent was then fed into an ESI interface operating in positive ion mode (spray voltage~5kV). A standard, data-dependent "triple-play" experiment in turn yielded full scan mass spectra (mass to charge ratio of parent ion), the second scan event was a zoom scan function to determine charge state of the observed ion, the third scan event was MS/MS of the parent ion to yield a fragmentation pattern for the peptide.

The MS/MS spectra were then analysed with the SEQUEST algorithm against a general FASTA protein database. This was then followed by more targeted searches against yeast, *E-coli* and *Aspergillus* FASTA protein databases

SEQUEST correlates uninterpreted MS/MS spectra of peptides with amino acid sequences from protein databases. SEQUEST will determine the amino acid sequence and thus the protein and organism that correspond to the mass spectrum being analysed. SEQUEST requires FASTA formatted databases. FASTA formatted databases are in ASCII text format, which means there is a single header/description line per sequence entry. This header line is denoted by the first character of the line being the greater than '>' sign.

5.3. Results

5.3.1. Protein concentration

Concentrations of estimated protein from each *S. lacrymans* replicate were calculated by extrapolation from the protein standard graph shown in figure 5.2. Typical protein concentrations for *S. lacrymans* controls ranged from 750-2300 µg/ml. These were higher than samples exposed to *T. aureoviride* VOCs then allowed to recover and samples grown after the VOCs had been removed which ranged from 190-340 µg/ml and 180-300µg/ml respectively. This represents inhibited growth in the former and apparently less dense growth after removal of the volatile stress.

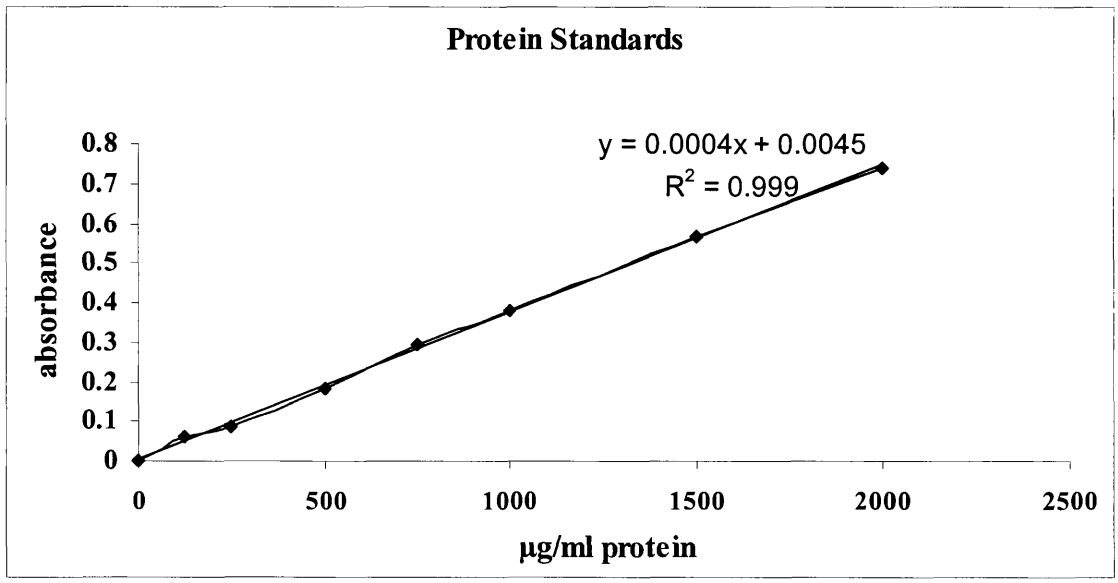


Figure 5.2. Protein standard graph from which unknown protein concentrations of samples were calculated.

5.3.2. SDS-PAGE

Protein profiles for *S. lacrymans* cultures exposed to *T. aureoviride* VOCs and allowed to recover and new growth after the VOC stress had been removed, are presented in figures 5.3a, 5.4a, 5.5a and 5.6a for *S. lacrymans* 12C, Forfar, H28 and BF050 respectively. Corresponding dendograms that show the percentage similarity between the 12-day-old control cultures and the 7-day-old controls (Chapter 4) are shown in figures 5.3b – 5.6b.

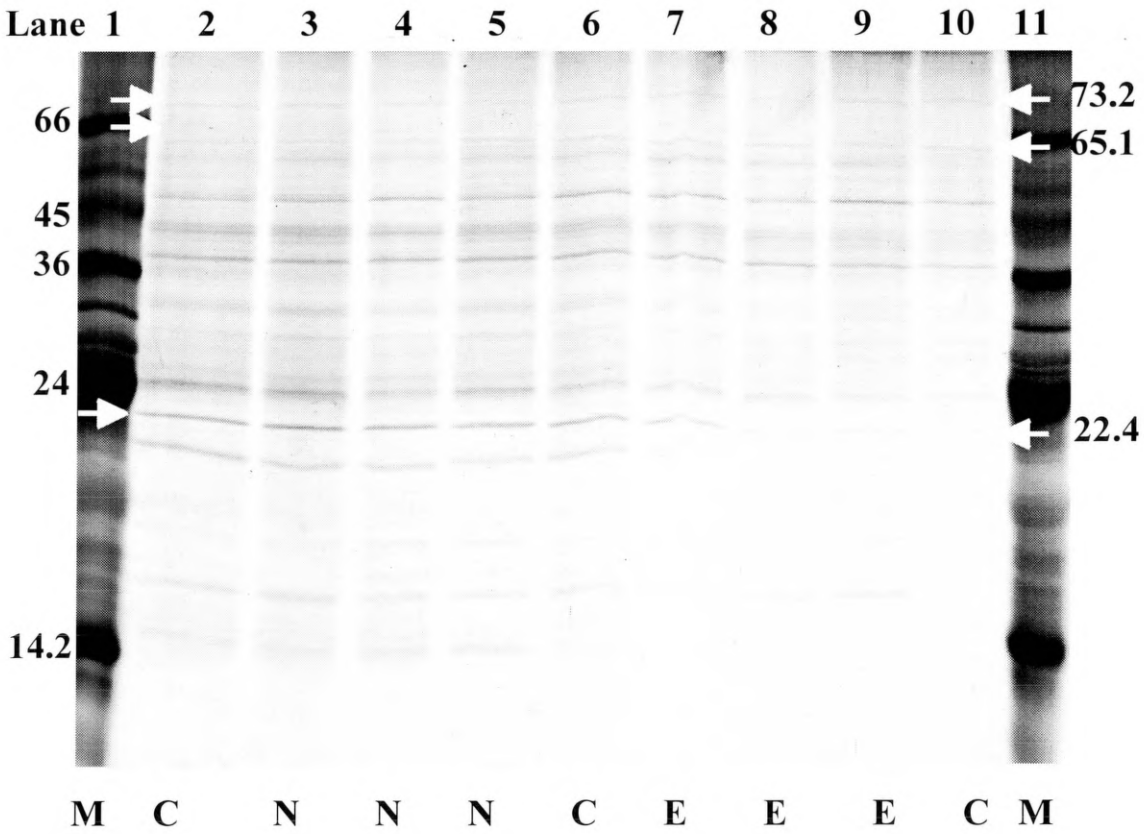


Figure 5.3a. Protein profile of *S. lacrymans* 12C; lanes 2,6, and 10 are the 12 day old control culture (C) which was not exposed to any antagonist; lanes 3-5 are the new growing edge of culture grown after removal of *T. aureoviride* VOCs (N); lanes 7-9 were exposed to *T. aureoviride* VOCs (E). Lanes 1 and 11 are the molecular weight markers. The MW of the proteins, in kilo-Daltons (kDa), which have reappeared after removal of *T. aureoviride* VOCs are highlighted down the right hand side of the gel with weights of the markers in kDa on the left.

The results from chapter 4 established that the proteins of molecular weights 22.4, 65.1 and 73.2 kDa were all inhibited by the VOCs from *T. aureoviride*, but were present in the 7-day-old control samples. Interestingly these 3 proteins are present in the samples that grew in the presence of the *T. aureoviride* VOCs, but from which the VOC was subsequently removed (Fig. 5.3a, lanes 7-9). These bands were also present in the new mycelium produced after removal of the VOC stress (Fig. 5.3a, lanes 3-5). It is clear therefore that the effect of the VOCs on protein production is only transient and that protein production resumes after removal of the VOC stress. In fact all protein bands are reproduced in both the controls (lanes 2, 6 and 10) and the samples exposed to the *T. aureoviride* VOCs as well as the samples newly grown after the VOC stress had been removed. Comparison of the 7-day-old control samples (Chapter 4) and the 12-day-old controls can be seen in Figure 5.3b, which shows 91% similarity between the 2 controls. The only difference between the protein profiles is that the protein of MW 85.0 kDa, which is produced by the 7-day-old control, but is no longer produced by the 12-day-old control samples.

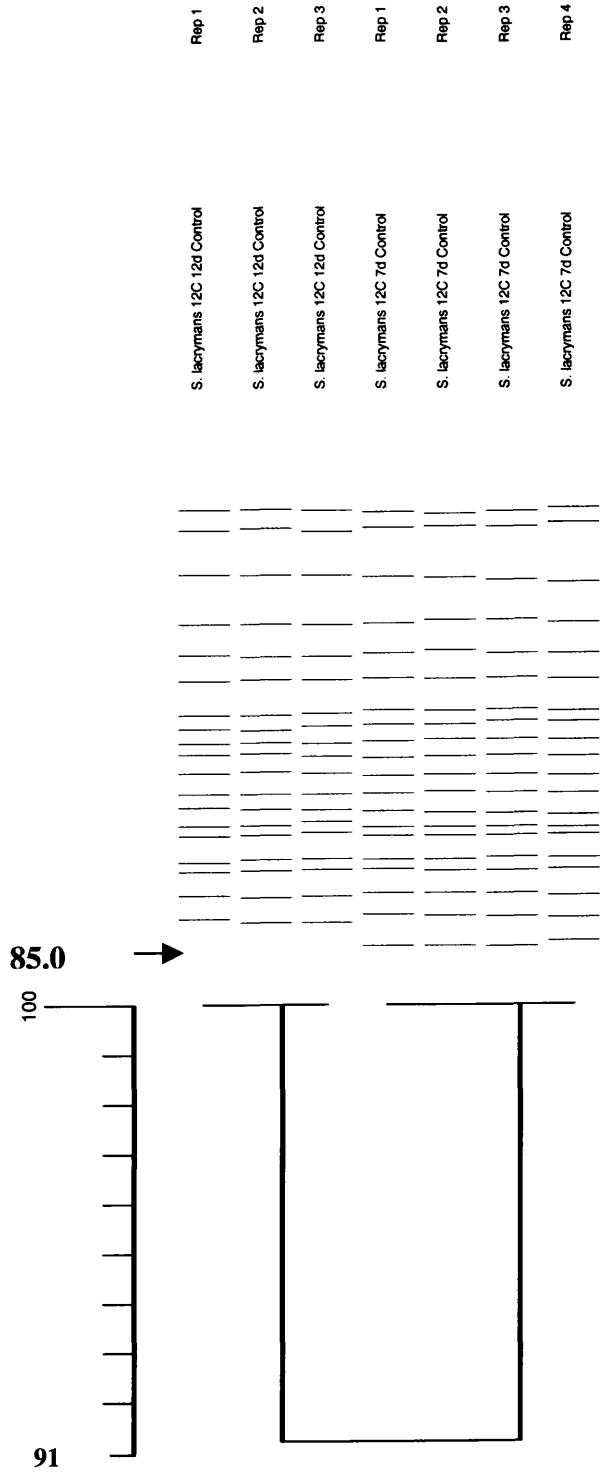


Figure 5.3b. Gelcompar generated dendogram of the bands produced by *S. lacrymans* 12C 7-day-old (7d control) and 12-day-old (12d control) control samples. The scale bar represents % similarity.

The protein profile for *S. lacrymans* Forfar is shown in figure 5.4a. The results from chapter 4 demonstrated that this isolate showed the least change in protein production of all of the *S. lacrymans* isolates when exposed to *T. aureoviride* VOCs. The VOCs from *T. aureoviride* had the same effect on the protein band at 22.4 kDa, with total inhibition being seen. This time however, the *T. aureoviride* VOCs only affected one other band at MW 29.1 kDa. Both these bands were present in the 7-day-old control samples. As can be seen from figure 5.4a the bands at 29.1 and 22.4 kDa are present in the samples grown in the presence of the *T. aureoviride* VOCs but subsequently removed (lanes 7-9) and the samples grown after the VOCs were removed (lanes 3-5). Again, in this case all protein bands are reproduced in both the controls (lanes 2, 6 and 10) and the samples exposed to the *T. aureoviride* VOCs as well as the samples newly grown after the VOC stress had been removed so are therefore 100% similar. Comparison of the 7-day-old control samples (Chapter 4) and the 12-day-old controls shows that there is 95% similarity between the 2 controls of different ages (Fig. 5.4b). As with *S. lacrymans* 12C, the only difference between the protein profiles is that the protein of MW 85.0 kDa produced by the 7-day-old control is no longer produced by the 12-day-old control samples.

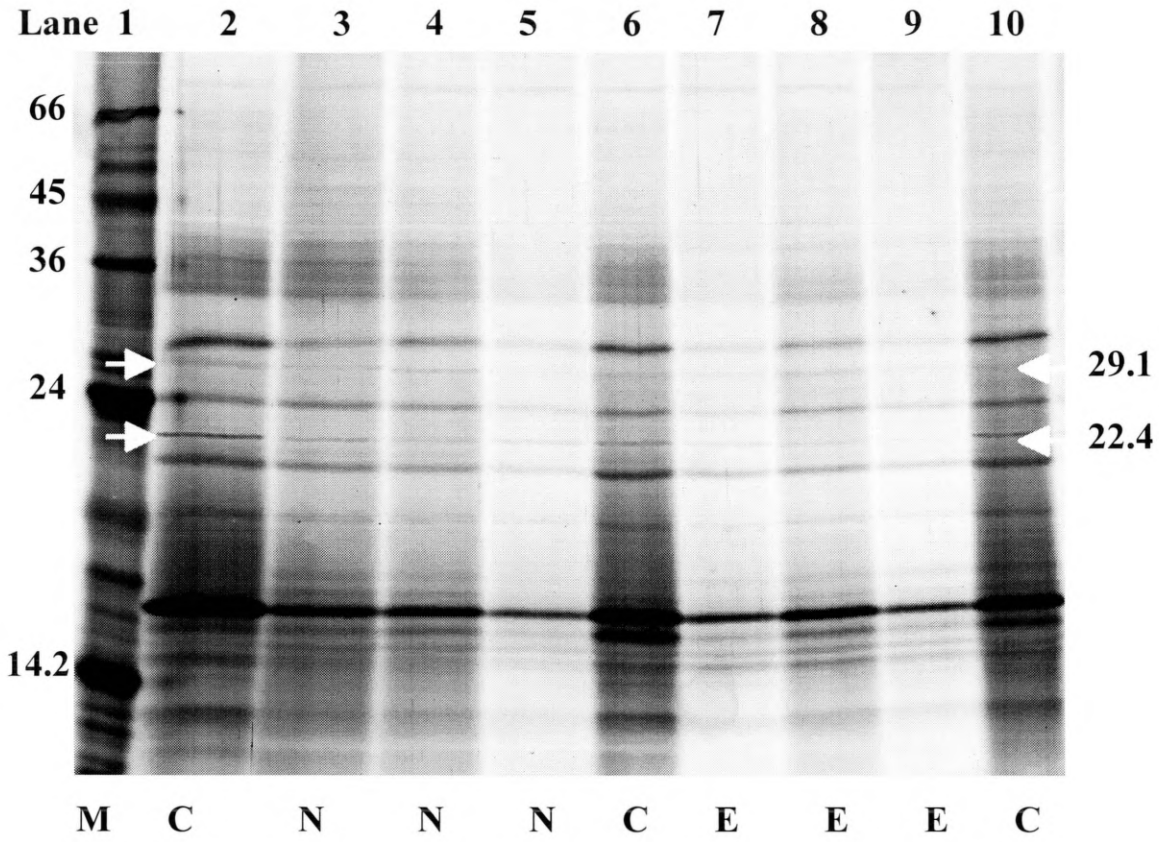


Figure 5.4a. Protein profile of *S. lacrymans* Forfar; lanes 2,6, and 10 are the 12 day old control culture (C) which was not exposed to any antagonist; lanes 3-5 are the new growing edge of culture grown after removal of *T. aureoviride* VOCs (N); lanes 7-9 were exposed to *T. aureoviride* VOCs (E). Lane 1 is the molecular weight marker. The MW of the proteins, in kilo-Daltons (kDa), which have reappeared after removal of *T. aureoviride* VOCs are highlighted down the right hand side of the gel with weights of the markers in kDa on the left.

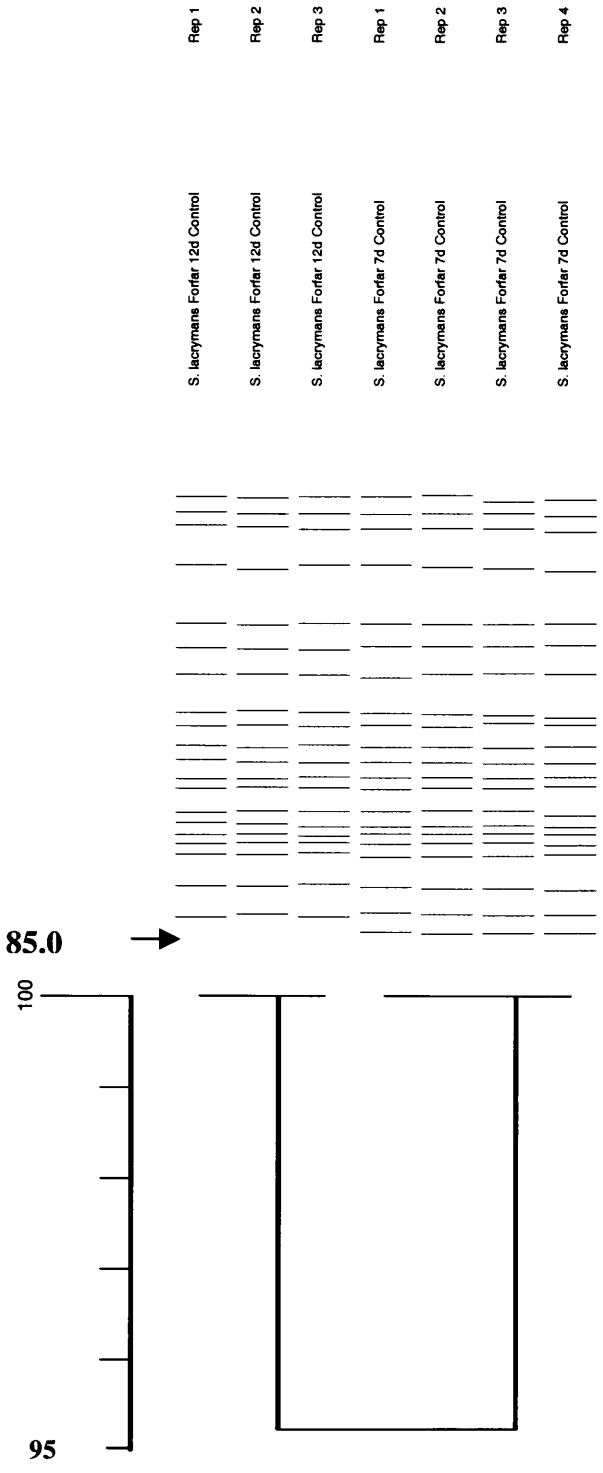


Figure 5.4b. Gelcompar generated dendogram of the bands produced by *S. lacrymans* Forfar 7-day-old (7d control) and 12-day-old (12d control) control samples. The scale bar represents % similarity.

The protein profile for *S. lacrymans* H28 is shown in figure 5.5a. This isolate showed the greatest changes in protein profile of the 4 *S. lacrymans* isolates when exposed to *T. aureoviride* VOCs (chapter 4). The bands at 65.1, 29.1 and 22.4 kDa were again totally inhibited by the VOCs from *T. aureoviride* and additionally the bands at MW 32.2 and 65.1 kDa were also inhibited. All 5 of these bands were present in the 7-day-old control samples (Chapter 4). All these bands are now present in the samples exposed to the *T. aureoviride* VOCs but with subsequent removal of the VOCs (Fig. 5.5a, lanes 7-9) and the samples of new growth after the VOCs had been removed (lanes 3-5). Protein production in all samples, including the 12-day-old controls (lanes 2, 6 and 10), is in fact identical. Comparison between the 7-day-old and 12-day-old control samples shows that bands of MW 79.3 and 85.0 kDa, which are present in the 7-day-old controls, are no longer produced in the 12-day-old control samples (Fig. 5.5b). The 7-day-old and 12-day-old controls are 88% similar (Fig. 5.5b).

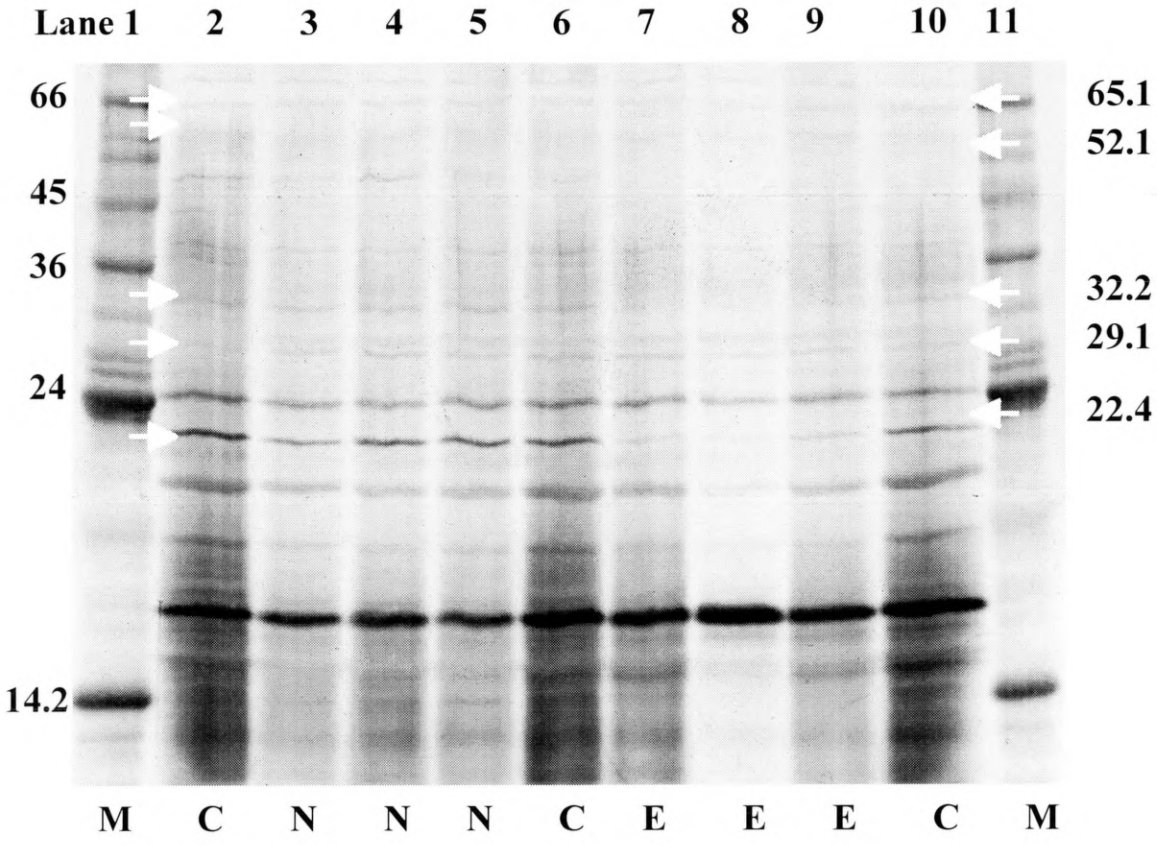


Figure 5.5a. Protein profile of *S. lacrymans* H28; lanes 2,6, and 10 are the 12 day old control culture (C) which was not exposed to any antagonist; lanes 3-5 are the new growing edge of culture grown after removal of *T. aureoviride* VOCs (N); lanes 7-9 were exposed to *T. aureoviride* VOCs (E). Lanes 1 and 11 are the molecular weight markers. The MW of the proteins, in kilo-Daltons (kDa), which have reappeared after removal of *T. aureoviride* VOCs are highlighted down the right hand side of the gel with weights of the markers in kDa on the left.

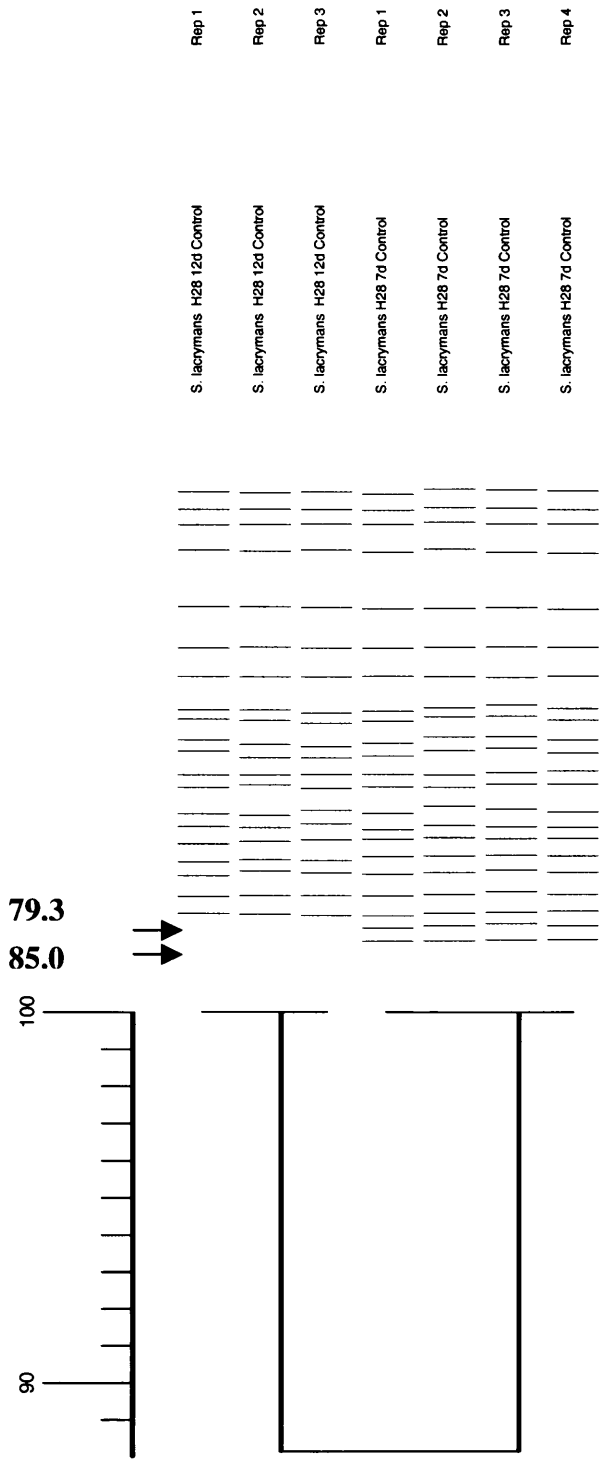


Figure 5.5b. Gelcompar generated dendogram of the bands produced by *S. lacrymans* H28 7-day-old (7d control) and 12-day-old (12d control) control samples. The scale bar represents % similarity.

The protein profile for *S. lacrymans* BF050 is shown in figure 5.6a. In this isolate the VOCs from *T. aureoviride* again inhibited the production of the proteins of MW 22.4, 73.2 and 67.5 kDa (Chapter 4). Production of these proteins was detected in the 7-day-old controls (Chapter 4) and again can be seen in the 12-day-old control samples (Fig 5.6a, lanes 2,6 and 10). The results from figure 5.6a demonstrate that production of all 3 of these proteins has resumed in the samples previously exposed to the VOCs (lanes 7-9) and the new growth after the *T. aureoviride* VOCs had been removed (lanes 3-5). Protein production is now identical in all samples. Comparison between the 7-day-old and 12-day-old control samples is shown in Figure 5.6b. The 2 controls are 82% similar with the bands at MW 79.3 and 85.0 kDa being produced by the 7-day-old controls, but not the 12-day-old control samples (Fig. 5.6b).

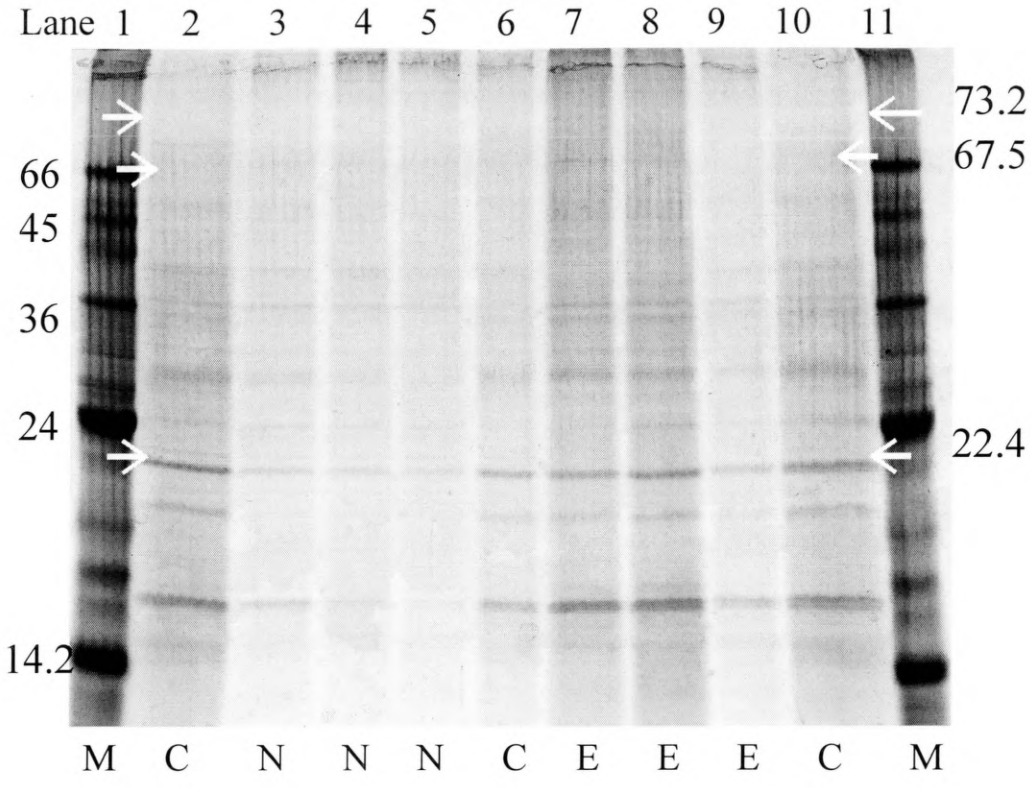


Figure 5.6a. Protein profile of *S. lacrymans* BF050; lanes 2,6, and 10 are the 12 day old control culture (C) which was not exposed to any antagonist; lanes 3-5 are the new growing edge of culture grown after removal of *T. aureoviride* VOCs (N); lanes 7-9 were exposed to *T. aureoviride* VOCs (E). Lanes 1 and 11 are the molecular weight markers. The MW of the proteins, in kilo-Daltons (kDa), which have reappeared after removal of *T. aureoviride* VOCs are highlighted down the right hand side of the gel with weights of the markers in kDa on the left.

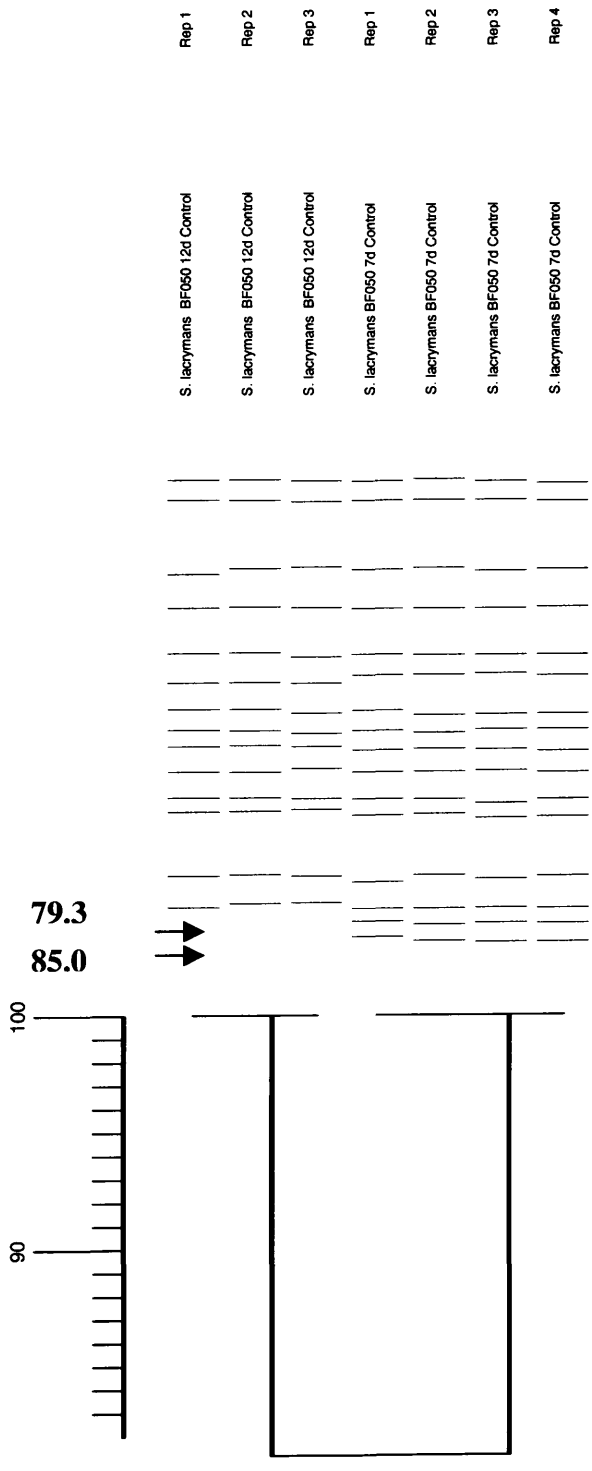


Figure 5.6b. Gelcompar generated dendogram of the bands produced by *S. lacrymans* BF050 7-day-old (7d control) and 12-day-old (12d control) control samples. The scale bar represents % similarity.

5.3.3. Electropspray ionisation mass spectrometry

Figure 5.7 is the MS/MS of the parent ion at retention time 47.62 minutes for the internal standard Alpha haemoglobin. The chromatogram and the MS/MS spectra for the parent ions at retention time 27.31minutes for the unidentified protein of MW 22.4 kDa are shown in Figure 5.8a and 5.8b respectively.

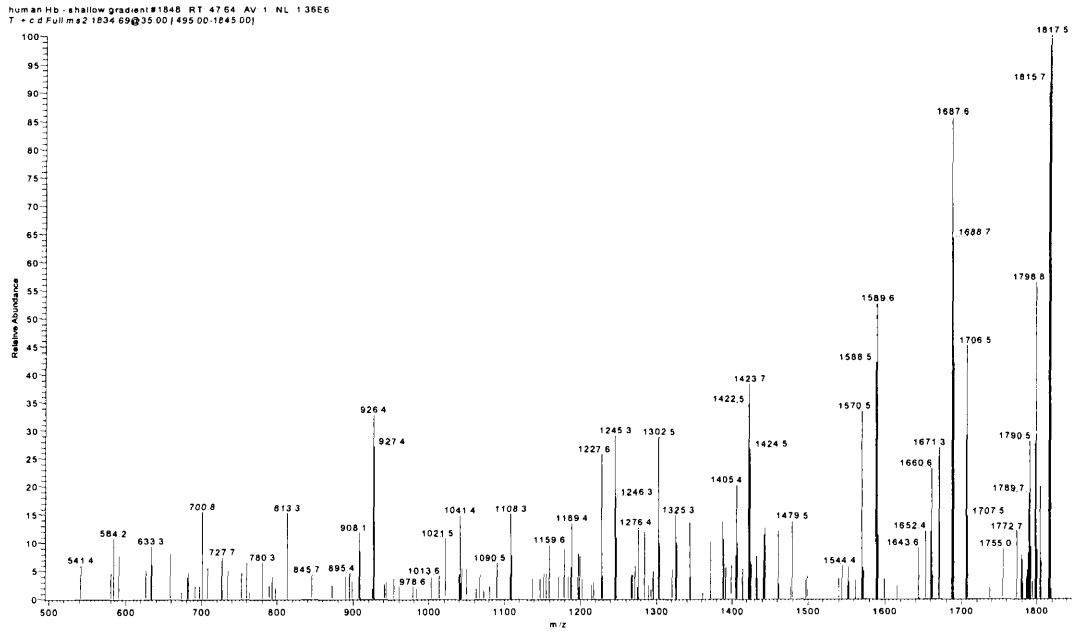


Figure 5.7. MS/MS spectra of ion at retention time 47.62 minutes.

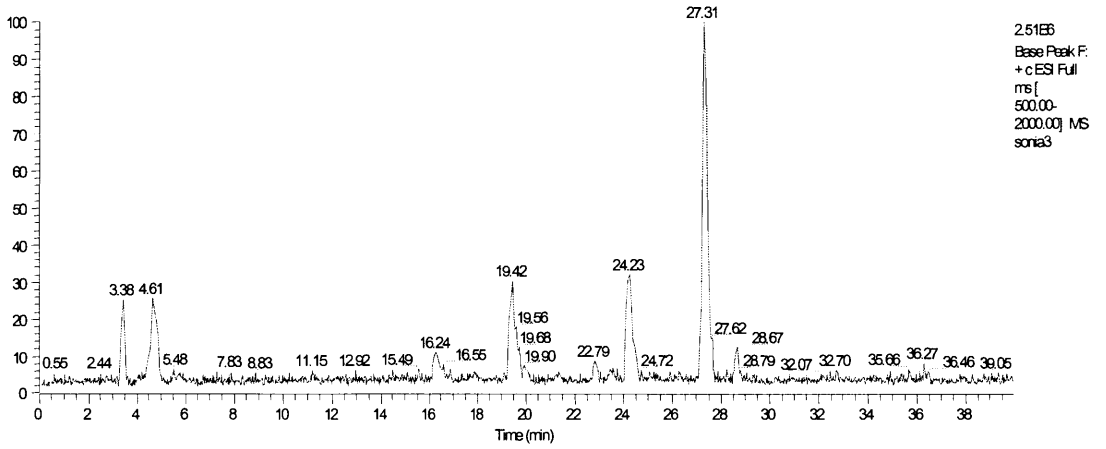


Figure 5.8a. Chromatogram of excised and digested protein band. Y-axis is relative abundance shown as percentage of the largest peak.

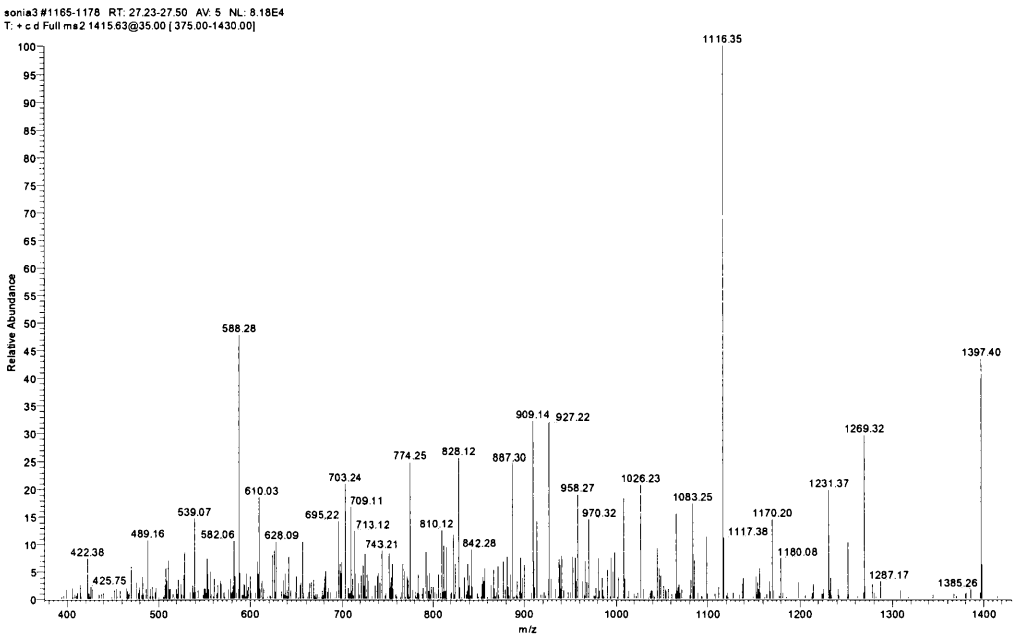


Figure 5.8b. MS/MS spectrum of ion at retention time 27.31minutes.

Using these MS/MS spectra SEQUEST in turn searches the FASTA protein databases for a match for the sequences of each fragment of the peptide. It then combines any sequences matches, which are from the same protein, to a give a percentage (by amino acid content) of the protein from the database covered by the fragments of the peptide. The following 5 fragment sequences of the ion at retention 47.62 minutes accounted for 54% of protein coverage by amino acid content of the protein identified as Human haemoglobin alpha chain (Fig. 5.9). This is considered a positive match using this form of protein identification.

a)

Position	**Sequence**
33 - 41	**MFLSFPTTK**
42 - 57	**TYFPHFDLSHGSAQVK**
62 - 91	**KVADALTNAVAHVDDMPNALSALSDLHAHK**
92 - 100	**LRVDPVNFK**
129 - 140	**FLASVSTVLTSK**

b) HUMAN HEMOGLOBIN ALPHA CHAIN

MVLSPADKTN VKAAWGKVGA HAGEYGAEAL ER**MFLSFPTT KTYFPHFDLS HGSAQVK**GHG K**KVADALTNA VAHVDDMPNA LSALSDLHAH KLRVDPVNFK** LLSHCLLVTL AAHLPAEFTP AVHASLDK**FL ASVSTVLTSK** YR

Figure 5.9. Peptide fragment sequences from trypsin digest (a) which correspond to sequences present in Alpha haemoglobin (b).

The search of the databases for the unknown protein only matched one sequence from any protein irrespective of whether the yeast, *E. coli* or *Aspergillus* databases were searched. This meant there was no logical match for the unidentified protein as there was never more than 4 % coverage by amino acid content of any protein identified (Table 5.1).

Peptide Sequence	Identification of Protein	% coverage	Protein Database
DEAVHGYYIGYK	Ribonucleoside-diphosphate reductase	3.8	*Ecoli*
GGSVRAVLSLQSLK	Cell division control protein	2.7	Yeast
SANVLDVSKLK	GTPase-activating protein	1.7	Yeast

Table 5.1. Top three matches obtained from the database searches for the unidentified protein at 22.4 kDa.

5.4. Discussion

The results presented here show that production of the proteins identified in chapter 4 as being inhibited by *T. aureoviride* has resumed in mycelia allowed to recover after grown under the influence of *T. aureoviride* VOCs. New growth after removal of the VOC stress also produced these inhibited proteins. This demonstrates that even though the *Trichoderma* VOCs inhibit growth and protein production, once this VOC stress is removed protein production returns to normal in the previously inhibited area of growth and is the same as the protein production in the new area of growth. This agrees with previous reports where after removal of environmental stresses from fungi and bacteria normal protein production gradually resumes (DiDomenico *et al.*, 1982; Plesofsky-Vig and Brambl, 1985).

The 7-day-old and 12-day-old control samples were very similar except that in all 4 isolates the band at 85.0 kDa was only present in the 7-day-old culture and for *S. lacrymans* H28 and BF050 the band at 79.3 kDa was also only present in the 7-day-old culture (This latter protein was not detected in either of the controls of 12C and Forfar). The intensity of both of these bands was faint in the 7-day-controls which suggests that neither protein was produced in large amounts and may therefore not be essential to the survival of *S. lacrymans*. It is possible that they could be involved in the early stages of mycelial development, which is why they are not produced in the 12-day-old culture and are present in small quantities in the 7-day-old culture. Vigrow *et al.* (1991a) demonstrated that antigenic profiles revealed differences between old and juvenile regions of *S. lacrymans* indicating that these may represent 2 different morphological types of mycelium, consistent with a programmed degradation of proteins late in the organisms life cycle prior to nitrogen re-use.

It is also interesting to note the difference in the growth morphology between the mycelia grown under exposure to the VOCs and allowed to recover and the new growth after the VOCs had been removed (Fig. 5.1). This growth resembles the growth referred to as point growth (Coggins *et al.*, 1980), where under conditions less favourable for growth surface mycelium extends from discrete points within and at the edge of the colony at a faster rate than the parent mycelium. Newly produced surface mycelium not only has a greater growth rate than the original mycelium but also has a lower density of hyphae. When growth conditions become less favourable, development of particular hyphae of the surface mycelium is triggered. This growth leads to a stimulation of growth and branching of single hyphae, the fan-like spread of surface mycelium and in a manner similar to the formation of strands, the differentiation of mycelium into small bundles. The growth after removal of VOC stress was less dense than growth during exposure to *Trichoderma* VOCs, which is a typical characteristic of point growth, this suggests that either the quantity or functionality of one or a number of proteins have been reduced. The protein of MW 22.4 kDa was clearly produced in lesser amounts when *S. lacrymans* was exposed to *T. viride* compared with the control samples (Chapter 4, section 4.3.2, Fig. 4.6a) and this might be occurring with other proteins as well. To establish whether this effect on growth morphology was transient or whether such effects on proteins involved in growth morphology was permanent, a plug of mycelia could be removed from the area of less dense growth and subcultured onto fresh malt extract. Figures 5.3a – 5.6a show however that the protein profiles for each of these two areas after removal of the VOC stress are identical. This shows no essential proteins are missing to account for this difference in growth morphology. This may indicate that, quantitatively, protein production has not returned to the levels prior to VOC exposure (controls). This could be assessed in the future by running known concentrations of MW markers on a gel

which could be quantitatively compared with the intensity of proteins in the test and control samples that are known to be responsible for growth and development.

Although it might be possible that the changes in protein profiles could be a result of the proteins being denatured by the VOCs, this is unlikely since the majority of the proteins are unaffected by exposure. It appears rather that the volatile secondary metabolites act on the basic expression of proteins by the fungus rather than interfering with enzyme function after synthesis. This is supported by the fact that protein bands that were missing in profiles from mycelia exposed to inhibitory VOCs reappear once the source of the VOCs is removed and continue to be produced in the new growth mycelia thereafter, although not in the same quantities. This experiment has therefore shown that VOCs produced by a potential biological control agent can have a regulatory effect on a target fungus (in this case *S. lacrymans)* at the very basic functional level of protein synthesis.

If volatile secondary metabolites can travel over distance through a substrate and enhance the status of one organism by affecting the physiology of competitor organisms they may well have a critical role in biological control systems against wood decay. It is probable that volatile secondary metabolites may play an important role during the evolution of microorganisms in the context of community, population and functional dynamics in wooden structures. This work establishes that since VOCs have an effect at the molecular level they are likely to be an important regulatory factor determining the inter-relationship between *Trichoderma* spp. and *S. lacrymans* in wood. The development of successful strategies for biological control requires knowledge of the biological control agents, the mechanisms involved in the biological control process and the effect of environment on both. If these VOCs affect the growth of *S. lacrymans*

then it is likely that they will also affect other fungi in wood. The result that this would have on the population dynamics of fungi in wood is unknown. It may create a niche for other fungi uninhibited by the *Trichoderma* VOCs, which could still cause significant levels of decay. This in turn may have an adverse effect on the inter-relationship between *Trichoderma* spp. and *S. lacrymans*.

It is important to note that removal of the volatile stress allowed the recovery of the decay fungus. Previous work has reported both fungistatic and fungicidal effects of *Trichoderma* VOCs against wood decay fungi (Bruce *et al.*, 1984). This fungistatic action may highlight a limitation for their significance in the biological control of *S. lacrymans* where eradication of an existing *Serpula* infection may be required. A possibility however is integrated control. Using chemical and biological control measures together may increase the duration of the active disease control, as the 2 methods may act synergistically and provide an alternative system as chemical protectants that are effective under climatic conditions or levels of fungal decay in which the biological antagonist is less effective.

One protein of MW 22.4 kDa was selected for further investigation using ESI-MS. This protein was selected on the basis it appeared to be linked to mycelial development as the degree of mycelial growth inhibition appeared to be related to reduction in synthesis of this protein. Two replicate test samples and controls were randomly selected from the 4 *S. lacrymans* isolates for the initial test experiment to determine whether any results would be found. While excising and digesting this protein did result in a map of peptide masses, a search of the databases with the molecular weights of the peptides did not result in a match against any known protein in what could be considered to be closely matched organisms (Yeast, *E. coli* and *Aspergillus*). This is particularly

disappointing as this protein appears to be linked to growth development of the *S. lacrymans* it is likely to be at least similar to proteins with the same functionality in other related organisms. Failure to identify the protein may however be because the protein has not previously been identified and is therefore not entered in any database. As yet there are no protein databases specifically for *S. lacrymans*.

Further work is required to establish the exact nature of the proteins affected by the specific inhibitory VOCs. This could be achieved by identifying the amino acid sequence of the protein by stepwise chemical degradation of isolated polypeptides from the N-terminal known as Edman degradation (Figeys and Aebersold 1998), the amino acid sequence would then in turn be used to identify the protein. However, large amounts of the protein are required for this and proteins can be N-terminally blocked. As there were also species specific proteins affected by the *Trichoderma* VOCs it would also be interesting to further identify these protein bands whose production was inhibited and then resumed after the stress was removed. If any of these proteins are essential to the survival of *S. lacrymans* it may be possible to target them in other ways that may be effective against other wood decay fungi. If the target proteins are common to other wood decay fungi then identification may be useful for development of control strategies against a variety of organisms.

CHAPTER 6: Final discussion and conclusions

The major aim of this thesis was to assess the production of volatile organic compounds by *Trichoderma* species and evaluate their importance as a mechanism of control of *S. lacrymans*. In chapter 2 experiments were designed to examine volatile interaction between *Trichoderma* and *S. lacrymans* isolates. A range of known *Trichoderma* antagonists was screened against 4 culture collections of *S. lacrymans*. Particular attention was paid to the composition of media used as previous work has shown the importance of nutrient composition on effectiveness of biological control by *Trichoderma* isolates (Srinivasan *et al.*, 1992; Score, 1998). The interaction studies were carried out on a nutrient rich synthetic media, a minimal media designed to mimic the carbon : nitrogen ratio in Scots pine sawdust and on wood shavings. Results indicated that the growth of *S. lacrymans* isolates were significantly inhibited by VOCs produced by *Trichoderma* isolates, but different strains of *Trichoderma* gave varying levels of inhibition of *S. lacrymans*. For example, *T. harzianum* 25 grown on malt inhibited *S. lacrymans* 12C growth by 84% but only inhibited BF050 by 19%. All strains of *S. lacrymans* whose growth was highly significantly inhibited displayed yellow pigmentation indicative of a stress reaction and there appeared to be a critical level of around 50% inhibition before any pigmentation was observed.

The production of VOCs was also influenced by the nutrient composition of the *Trichoderma* growth media with generally greater inhibition being produced when *Trichoderma* isolates were cultured on nutrient rich substrate. For example, *S. lacrymans* Forfar growth was inhibited by *T. aureoviride* VOCs when grown on malt by 92%, but was only inhibited by 31% and 40% when the *T. aureoviride* was grown on the minimal and sawdust media, respectively. Interestingly *T. viride* T110 gave higher levels of inhibition when grown on sawdust rather than malt media for *S. lacrymans* H28 and FPRL 12C. Identification of the individual VOCs (Chapter 3) showed that this

was because *T. viride* T110 produced different quantities of a critical inhibitory VOC (2-heptanone) when grown on the sawdust. The majority of the levels of inhibition produced by the VOCs were still significant when the *Trichoderma* isolates were grown on the minimal and sawdust media.

Considering all media types and *Serpula* strains, the same 2 *Trichoderma* isolates, (*T. polysporum* and *T. aureoviride*) consistently produced the greatest levels of inhibition. However, the *S. lacrymans* isolates still showed different sensitivities to the VOCs produced by these 2 *Trichoderma* isolates. For example, on the malt extract *S. lacrymans* Forfar was inhibited by 92% by *T. aureoviride* IMI 91968 whereas *S. lacrymans* H28 was inhibited by 75%. In fact, H28 seemed to be the least susceptible to the VOCs when the *Trichoderma* isolates were grown on malt media, with the inhibition never being above 78%, whereas *S. lacrymans* Forfar appeared to be the most susceptible, with levels of inhibition reaching 92%. It may be that the Himalayan isolate is more tolerant to environmental changes so has more resistance than the other 3 strains when grown on malt extract rather than its natural substrate. White *et al.* (1997) have reported previously that wild Himalayan isolates are better suited to growing in a fluctuating or more stressful external environment and that building isolates have adapted for growth within the environmentally narrow building niche.

On all three media, *T. pseudokoningii* produced the lowest levels of inhibition against all *S. lacrymans* isolates with inhibition never being above 15%. There was even significant stimulation of BF050 on the minimal media by *T. pseudokoningii* as well as *T. koningii* and *T. viride* 70 and significant stimulation of Forfar on the minimal media by *T. viride* 70.

In conclusion, the inhibition of the *S. lacrymans* isolates was clearly dependent on the *Trichoderma* isolate, the composition of the media and the sensitivity of the *Serpula* strain, with growth inhibition consistently resulting in the production of a yellow pigmentation on the *Serpula* mycelium. It is clear from the results, if *Trichoderma* is to be successful in an industrial application as a biological control agent of *S. lacrymans* then variability of inhibition within strains of *Trichoderma*, the nutritional requirements for maximum inhibition and the different sensitivities of the *S. lacrymans* isolates have to be taken into account.

On the basis of the findings of chapter 2, three isolates were selected for further investigation. They were *T. aureoviride*, which gave significant levels of inhibition for all strains of *Serpula* when grown on all three media; *T. pseudokoningii*, which generally gave no significant levels of growth inhibition and *T. viride* 110, which was unusual in that it gave higher levels of inhibition when grown on sawdust rather than malt media for *S. lacrymans* H28 and 12C. The objective of chapter 3 was to examine the changes in VOC profiles when grown on different media and to identify individual VOCs produced by the *Trichoderma* isolates that are most likely to be responsible for the inhibition of the dry rot fungus. Volatiles were collected from the 3 *Trichoderma* isolates grown on the three media when the cultures were 7 days old. Bruce *et al.* (1996) found that the age of *T. aureoviride* cultures influenced the levels of inhibition of decay fungi and also influenced the VOC profile, with 7-14 day old cultures of *T. aureoviride* giving greatest inhibition of decay fungi, and was associated with an increase in the production of aldehydes and ketones. The analysis of VOC production by the three *Trichoderma* isolates in this study implicated 8 compounds, 2-dodecanone, 1,3 cyclohexadiene, cyclohexene, isobutanol, naphthalene 3-octanone, 2-nonanone and

2-heptanone that may be responsible for the inhibition of *S. lacrymans*. Interestingly half of the compounds identified were ketones.

It may be possible to apply these compounds as chemical fumigants of wooden structures. However, the health and safety implications of such a process makes this unlikely even if the compounds could be applied at low concentrations. Future studies could include testing the possibility of using these ketones as a strategy for wood preservation. This would determine the concentration of the chemical required for preservation and the toxicity of the compound at that concentration.

Compounds of particular interest were 3-octanone, 2-nonanone and 2-heptanone. All three of these compounds were produced by *T. aureoviride* on both malt media and sawdust. The greatest levels of inhibition were detected when *T. aureoviride* was grown on malt media and if *Trichoderma* is to be successful as a remedial treatment of dry rot in buildings the volatiles identified as active inhibitory compounds must be produced by the potential biocontrol agent in wood. Both 2-nonanone and 2-heptanone were produced in large amounts on the sawdust; 3-octanone however, was only produced in relatively small amounts. The amount of any volatile produced however, does not necessarily indicate its potency and in substrates such as wood where cell lumina can allow slow gaseous diffusion, VOCs released by resident microorganisms may build up and become toxic to other organisms that colonise the wood, whereas in a more diffuse substrate the VOC may be more easily dispersed. The greater amounts of 2-heptanone produced on the sawdust and minimal media by *T. viride* probably explain why higher levels of inhibition were seen for *S. lacrymans* H28 and 12C compared with the *T. aureoviride* on the same media. The fact that only *S. lacrymans* 12C and H28 showed this increase in inhibition again illustrates strain specificity with these two

isolates being more susceptible to 2-heptanone. *T. pseudokoningii* stimulated the growth of *S. lacrymans* BF050 when grown on minimal media and the results of the VOC production for *T. pseudokoningii* grown on the 3 media identified 5 of the 6 compounds significant for those treatments as alcohols. Humphris *et al.* (2001) previously reported that an alcohol (2-methyl-1-butanol) also stimulated the growth of the basidiomycete *P. placenta* at low concentrations.

It is clear from the analysis of VOCs reported here and their inhibitory and in rare cases stimulatory effects that VOC production by *Trichoderma* spp. can play a fundamental role in microbial ecology of ecosystems dominated by fungi. It is also clear that the best possible application will be to apply the *Trichoderma* species rather than the identified compounds as fumigants. For this to be achieved the specificity of application will need to be taken into consideration, as it has not been determined whether *Trichoderma* isolates produce different profiles of compounds, which may be more or less inhibitory, when grown on different wood species. Different wood species contain ranges in amino acids and/or extractives that can vary even between different parts of wood such as the heartwood and sapwood. In addition, it is not known what effect other fungi within wood would have on *Trichoderma* spp. and their ability to inhibit *Serpula*. Even if it was possible to inhibit *Serpula,* this niche could be filled with another wood decay fungus not inhibited by *Trichoderma*. Fortunately, however, *Serpula* in buildings occupies a unique niche, which means that it is unlikely that another fungus, as destructive as *S. lacrymans*, would fill this niche. For *Trichoderma* spp. to be effective as a biological control agent *in situ* it is necessary to also examine VOC profiles produced after *S. lacrymans* have been exposed to *Trichoderma* when grown on different wood species and determine whether the inhibitory compounds identified in chapter 3 were still being produced.

In conclusion, the results from chapter 3 identified 8 compounds which may be involved in the inhibition of growth of *S. lacrymans* and indicated that no single compound is involved in the inhibition of growth of *S. lacrymans* but rather a combination of 2 or more VOCs are responsible for the inhibitory effect. The range and quantity of volatiles produced is dependent on the *Trichoderma* isolate and the media on which it was growing. In addition and of great practical significance, the compounds produced by the most inhibitory treatment (*T. aureoviride* on malt media) were also produced on the sawdust.

The final objective of this project was to establish the mode of action of *Trichoderma* volatiles against *S. lacrymans* by assessing the effect of VOCs on extracellular enzyme activity and protein production by the dry rot fungus. After exposure of the *S. lacrymans* strains to all 9 *Trichoderma* isolates, the *Serpula* cultures were assayed for the production of 3 extracellular enzymes, tyrosinase, cellulase and peroxidase. The results of this experiment indicated that tyrosinase was not produced by *S. lacrymans* in either control plates or plates exposed to the VOCs produced by *Trichoderma*. Since yellow pigmentation was often detected in plates of *S. lacrymans* that had been inhibited it is unlikely that tyrosinase plays a role in this pigment production. This study has also shown that *S. lacrymans* produces cellulase and this enzyme was produced continuously by the *S. lacrymans* isolates and not affected by *Trichoderma* volatiles. If inhibitory VOCs were only fungistatic and also had no effect on the oxidative agent involved in the early stages of wood, then *S. lacrymans* may still be able to secrete cellulase enzymes and still be capable of degrading cellulose even if growth was inhibited. However, the reduction in *S. lacrymans* biomass due to the inhibition of growth by the *Trichoderma* VOCs would significantly decrease cellulase production. In this study the production of cellulase by *S. lacrymans* was only tested on malt extract,

future studies could examine the production of cellulase when both *Trichoderma* and *Serpula* are grown on a sawdust or wood based substrate.

Increased peroxidase production was detected in all *S. lacrymans* cultures that were inhibited by VOCs from *T. polysporum* IMI 206039, *T. aureoviride* IMI 91968, *T. harzianum* IMI 206040 and FY *Trichoderma* on malt extract agar and in *S. lacrymans* BF050 and Forfar when exposed to *T. viride* T110 and *S. lacrymans* 12C and H28 when exposed to *T. harzianum* 25. All these cultures showed growth inhibition above 50% and also displayed yellow pigmentation, which suggests that increased peroxidase production is linked to increased pigmentation associated with a stress reaction in the fungus. For future studies it would be interesting if *S. lacrymans* isolates were exposed to other ecological stresses (for example changes in temperature, pH or oxygenation) or exposed to the compounds identified in chapter 3 to determine whether the same yellow pigmentation can be detected and whether a similar increase in peroxidase production can be detected.

S. lacrymans protein profiles were examined after the 4 isolates had been exposed to the VOCs from *T. aureoviride*, *T. viride* 110 and *T. pseudokoningii.* The results demonstrated for the first time that production of some fungal proteins was inhibited in the presence of *Trichoderma* VOCs from *T. aureoviride and T. viride,* but not by those of *T. pseudokoningii*. While differences and changes to protein profiles after exposure to inhibitory *Trichoderma* VOCs were recorded for each of the *S. lacrymans* strains, the protein at MW 22.4 kDa showed the same response for all 4 of the *Serpula* isolates. Production of this protein correlated well against growth inhibition with production of this protein completely inhibited by the VOCs from *T. aureoviride*, which gave the

greatest levels of inhibition of the *S. lacrymans* isolates. The protein was unaffected by VOCs from *T. pseudokoningii*, which gave very little or no inhibition of growth of *S. lacrymans*, while VOCs from *T. viride* (which gave on average between 35-50% inhibition of growth) caused a marked reduction in synthesis of the 22.4kDa protein. This suggests that this protein may be linked to mycelia development in *Serpula*. Analysis of the inhibited protein at 22.4 kDa by electrospray ionization did not result in the successful absolute identification of this protein. The response of the *S. lacrymans* isolates was species specific to both the *Serpula* isolate and the *Trichoderma* strain, with *S. lacrymans* H28 showing the greatest change in protein expression and *S. lacrymans* Forfar showing the least change. This is despite the fact that this later strain showed greatest growth inhibition when exposed to *Trichoderma* VOCs and H28 showed the least growth inhibition. However, the fact that H28 is less sensitive might imply that there would be a need for greater changes at the molecular level for this strain to overcome such VOC stresses. No extra bands appeared in the protein profiles of *S. lacrymans* isolates exposed to *Trichoderma* VOCs when compared with control samples, therefore changes produced may represent diminution in production of non-essential or 'housekeeping' proteins. Future studies should therefore concentrate on assessment of quantitative changes in production of protein bands under exposure to *Trichoderma* VOCs. Increases in band intensities may illustrate the increased expression of proteins such as peroxidase to overcome the toxic effects of the VOCs. Fundamental to this is the need to identify the proteins associated with the survival of the fungus. Melin *et al.* (2002) found that an antibiotic produced by *Streptomyces* altered the production of proteins in *A. nidulans* by either increasing or decreasing production rather than simply switching production on or off. Cultures exposed to non-inhibitory VOCs, such as *T. pseudokoningii*, showed no changes in protein production.

There must be a critical level of inhibition, for example, 35%, before any changes can be observed in *S. lacrymans*. Before this toxic threshold is reached, there is no obvious response in *Serpula*, no yellowing, no increase in enzyme levels and no changes in the number of proteins produced.

Removal of the antagonistic stress of *T. aureoviride* VOCs demonstrated that the inhibition of *S. lacrymans* growth was transient and that the VOC action was fungistatic rather than fungicidal. Not only did growth resume but also production of all proteins previously inhibited also resumed. This illustrates a potential risk for the use of *Trichoderma* spp. in the remedial treatment in buildings, areas of *S. lacrymans* not continually exposed to the critical level of VOCs necessary for inhibition may be capable of recovering and continuing to decay timber. *Serpula* species are also capable of producing strands and it was not established whether the VOCs had any effect on strand formation in *Serpula*. Therefore, while the growth of the *Serpula* may be inhibited, strand formation would allow the colony to traverse away from the volatile stress and attack fresh timber. This study has not assessed the effect of *Trichoderma* volatiles on this unique type of mycelium. However, no strand formation was detected during the volatile interactions and strand formation is generally thought to be triggered by nutrient exhaustion rather than abiotic stress and is involved in the translocation of water and nutrients over non-nutritional surfaces. After removal of the antagonistic stress however, it was evident that the production of inhibitory VOCs had altered the growth morphology of *S. lacrymans* with point growth observed (Coggins *et al.*, 1980). This third type of growth occurs under conditions less favourable for growth where surface mycelium extends from discrete points within and at the edge of the colony at a faster rate than the parent mycelium. Newly produced surface mycelium not only has a greater growth rate than the original mycelium but also has a lower density of hyphae.

One advantage for any potential biocontrol agent is that if the leading hyphae, whilst utilizing the nutrient produced from the parent mycelium, do not reach a new nutrient source, the point growth mycelium will rapidly lose viability.

Biological control could be a highly effective and inexpensive way of preserving buildings that are falling into disrepair due to lack of use and maintenance. By fully understanding the mode of action whereby *Trichoderma* spp. antagonise *S. lacrymans* new control methods may well be identified, whether based on live organisms, metabolites from such organisms or specific chemicals which can induce or mimic effects which occur during lethal interactions. The results of this thesis have demonstrated for the first time the mode of antagonism of *Trichoderma* VOCs against *S. lacrymans*, with inhibitory VOCs both up and down regulating protein production in *S. lacrymans*. While *S. lacrymans* isolates were exposed to *Trichoderma* VOCs, protein production was down regulated, and removal of the antagonistic stress resulted in up regulation of specific fungal proteins. Exposing *S. lacrymans* to the compounds identified in chapter 3 may induce a more extreme reaction in the fungus, which may well be fungicidal rather than fungistatic and allow identification of critical proteins that are essential to the survival of *Serpula* or involved in the decay process. By targeting these key proteins and finding a non-transient effect, this would eliminate the unpredictability of specificity of any potential biological control agent and also the influence of other VOCs on the biocontrol agent.

6.1. Future Work

Based on the results presented in this thesis, the following areas have been identified for potential future research:

- An investigation of the effect of *Trichoderma* VOCs on production of cellulase and oxalic acid, (the oxidative agent implicated in the early stages of *S. lacrymans* decay) when both *Trichoderma* and *Serpula* are grown together on a sawdust or wood based substrate.

- Testing the individual inhibitory effects of those compounds identified in chapter 3 in pure form, to determine the concentration of these chemicals against *S. lacrymans*. This would determine whether there is a possibility of using such wood preservatives at environmentally acceptable concentrations.

- For *Trichoderma* spp. to be effective as a biological control agent *in situ* it will be necessary to examine and verify whether the inhibitory compounds identified in chapter 3 will still be produced when *Trichoderma* is grown on different wood species and in the direct presence of *S. lacrymans*. Both these factors may alter the profile of VOCs produced by *Trichoderma*, leading to the fungus becoming more or less inhibitory.

- Exposing *S. lacrymans* isolates to other ecological stresses (for example changes in temperature, pH or oxygenation) or to the individual VOCs identified in chapter 3. This would establish whether the same stress reaction (yellow pigmentation)

observed in chapter 2 was produced and also whether similar increases in peroxidase production could also be detected.

- Since all protein analysis in this project has been qualitative, it would be beneficial to carry out quantitative assessment of changes in protein production by *S. lacrymans* after exposure to *Trichoderma* VOCs. Increases in band intensities may illustrate the increased expression of proteins such as peroxidase to overcome the toxic effects of the VOCs and decreases may indicate reduction in non-essential or 'housekeeping' proteins

- Furthermore, studies could be carried out to examine whether exposure to individual VOCs or other environmental stresses produced similar changes to protein profiles of *S. lacrymans*. It will be invaluable using Edman degradation to identify the protein of MW 22.4 kDa, which is clearly linked to mycelium development. This would help to establish whether this and other critical proteins are essential to the survival of Serpula or involved in the wood decay process and whether they can be targeted by individual VOCs.

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APPENDIX A

Appendix A

A 1.1. Moisture content

Ten 4g samples were removed from the bag of sawdust and after autoclaving were weighed to give the wet weight. The sawdust was then dried at 100° C for 2 hours and re-weighed to give the oven dry weight. The moisture content of the 10 samples of sawdust was then calculated using the formula below:

$$\frac{\text{Wet weight} - \text{Dry weight}}{\text{Wet Weight}} \times \frac{100}{1} = \%\ \text{moisture content}$$

Sample	Wet Weight (g)	Dry Weight (g)	% MC
1	8.752	7.213	17.58
2	8.515	7.088	16.76
3	8.669	7.094	18.17
4	8.66	7.085	18.19
5	8.364	7.018	16.09
6	8.755	7.051	19.46
7	8.53	7.099	16.78
8	8.524	7.125	16.41
9	8.631	7.123	17.47
10	8.505	7.106	16.45

This gave an average moisture content of 17%. The following calculation was carried out to determine how much water to add to the 4g of sawdust to obtain a MC of 120% :

$$4 \times \frac{120 - 17}{100} = 8\text{ml of water needed to bring the MC to 120\%}$$

A 1.2. ANOVA output for all 4 *S. lacrymans* strains exposed to 9 *Trichoderma* isolates grown on 3 media.

1.2.1. *S. lacrymans* 12C exposed to *Trichoderma* isolates grown on malt

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	160.78	53.593333	9.3258333
T. aureoviride	3	28.8643	9.6214335	15.121433

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	2900.292	1	2900.292	237.26922	0.0001036	7.7086497
Within Groups	48.894533	4	12.223633			
Total	2949.1865	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	160.78	53.593333	9.3258333
FY Trichoderma	3	16.575751	5.5252504	14.525321

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	3465.8109	1	3465.8109	290.61997	6.944E-05	7.7086497
Within Groups	47.702309	4	11.925577			
Total	3513.5132	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	160.78	53.593333	9.3258333
***T. harzianum* 25**	3	26.73817	8.9127234	22.341767

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	2994.5354	1	2994.5354	189.12297	0.000162	7.7086497
Within Groups	63.3352	4	15.8338			
Total	3057.8706	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	160.78	53.593333	9.3258333
***T. harzianum* 206040**	3	28.413294	9.4710981	7.5118744

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	2920.1575	1	2920.1575	346.85926	4.893E-05	7.7086497
Within Groups	33.675415	4	8.4188539			
Total	2953.8329	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	160.78	53.593333	9.3258333
T. koningii	3	81.634378	27.211459	45.386677

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1044.0049	1	1044.0049	38.163298	0.0034879	7.7086497
Within Groups	109.42502	4	27.356255			
Total	1153.4299	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	160.78	53.593333	9.3258333
T. polysporum	3	17.724408	5.9081359	1.4676249

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	3410.8171	1	3410.8171	632.01561	1.486E-05	7.7086497
Within Groups	21.586917	4	5.3967291			
Total	3432.404	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	160.78	53.593333	9.3258333
T. pseudokoningii	3	170.12	56.706667	4.4558333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	14.539267	1	14.539267	2.1099432	0.2199949	7.7086497
Within Groups	27.563333	4	6.8908333			
Total	42.1026	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	160.78	53.593333	9.3258333
***T. viride* 70**	3	121.43852	40.479505	6.165484

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	257.95873	1	257.95873	33.303654	0.0044758	7.7086497
Within Groups	30.982635	4	7.7456587			
Total	288.94137	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	160.78	53.593333	9.3258333
***T. viride* 110**	3	96.009305	32.003102	9.7748304

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	699.20715	1	699.20715	73.212864	0.0010243	7.7086497
Within Groups	38.201327	4	9.5503319			
Total	737.40847	5				

1.2.1.2 *S. lacrymans* 12C exposed to *Trichoderma* isolates grown on sawdust

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	168.65	56.216667	5.7002333
T. aureoviride	3	76.39845	25.46615	5.4328635

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	1418.3914	1	1418.3914	254.80626	9.004E-05	7.7086497
Within Groups	22.266194	4	5.5665484			
Total	1440.6576	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	168.65	56.216667	5.7002333
FY Trichoderma	3	134.49838	44.832792	2.8977864

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	194.38892	1	194.38892	45.217136	0.0025473	7.7086497
Within Groups	17.196039	4	4.2990098			
Total	211.58495	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	168.65	56.216667	5.7002333
***T. harzianum* 25**	3	126.90379	42.301264	1.3467083

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	290.45765	1	290.45765	82.435096	0.0015824	7.7086497
Within Groups	14.093883	4	3.5234708			
Total	304.55153	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	168.65	56.216667	5.7002333
***T. harzianum* 206040**	3	151.42521	50.475071	11.45463

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	49.448879	1	49.448879	5.7649982	0.0742724	7.7086497
Within Groups	34.309728	4	8.5774319			
Total	83.758607	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	168.65	56.216667	5.7002333
T. koningii	3	156.85574	52.285248	7.2840594

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	23.184082	1	23.184082	3.5710966	0.1317909	7.7086497
Within Groups	25.968585	4	6.4921463			
Total	49.152667	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	168.65	56.216667	5.7002333
T. polysporum	3	72.808992	24.269664	17.960389

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	1530.9165	1	1530.9165	129.40627	0.0003406	7.7086497
Within Groups	47.321244	4	11.830311			
Total	1578.2377	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	168.65	56.216667	5.7002333
T. pseudokoningii	3	176.56531	58.855102	9.4762741

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	10.442013	1	10.442013	1.3760759	0.3058577	7.7086497
Within Groups	30.353015	4	7.5882537			
Total	40.795028	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	168.65	56.216667	5.7002333
***T. viride* 70**	3	142.00892	47.336307	8.1760579

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	118.29118	1	118.29118	17.049394	0.0145046	7.7086497
Within Groups	27.752582	4	6.9381456			
Total	146.04376	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	168.65	56.216667	5.7002333
***T. viride* 110**	3	66.970915	22.323638	2.2228382

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	1723.1061	1	1723.1061	434.95911	3.123E-05	7.7086497
Within Groups	15.846143	4	3.9615358			
Total	1738.9522	5				

1.2.2. S. *lacrymans* Forfar exposed to *Trichoderma* isolates grown on malt.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	164.48	54.826667	2.5125333
T. aureoviride	3	13.87	4.6233333	0.7633333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	3780.562	1	3780.562	2308.1294	1.123E-06	7.7086497
Within Groups	6.5517333	4	1.6379333			
Total	3787.1138	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	164.48	54.826667	2.5125333
FY Trichoderma	3	13.79	4.5966667	12.586133

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	3784.5794	1	3784.5794	501.31305	2.356E-05	7.7086497
Within Groups	30.197333	4	7.5493333			
Total	3814.7767	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	164.48	54.826667	2.5125333
***T. harzianum* 25**	3	108.66	36.22	34.7269

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	519.31207	1	519.31207	27.890439	0.0061654	7.7086497
Within Groups	74.478867	4	18.619717			
Total	593.79093	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	164.48	54.826667	2.5125333
***T. harzianum* 206040**	3	23.51	7.8366667	5.2622333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	3312.0902	1	3312.0902	852.01017	8.201E-06	7.7086497
Within Groups	15.549533	4	3.8873833			
Total	3327.6397	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	164.48	54.826667	2.5125333
T. koningii	3	103.8	34.6	16.2787

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	613.67707	1	613.67707	65.315252	0.0012736	7.7086497
Within Groups	37.582467	4	9.3956167			
Total	651.25953	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	164.48	54.826667	2.5125333
T. polysporum	3	16.02	5.34	5.6901

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	3673.3953	1	3673.3953	895.66243	7.424E-06	7.7086497
Within Groups	16.405267	4	4.1013167			
Total	3689.8005	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	164.48	54.826667	2.5125333
T. pseudokoningii	3	139.77	46.59	31.8081

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	101.76402	1	101.76402	5.9301946	0.0715781	7.7086497
Within Groups	68.641267	4	17.160317			
Total	170.40528	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	164.48	54.826667	2.5125333
***T. viride* 70**	3	139.05	46.35	10.8381

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	107.78082	1	107.78082	16.146173	0.0158877	7.7086497
Within Groups	26.701267	4	6.6753167			
Total	134.48208	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	164.48	54.826667	2.5125333
***T. viride* 110**	3	75.5	25.166667	32.662233

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	1319.5734	1	1319.5734	75.029547	0.0009774	7.7086497
Within Groups	70.349533	4	17.587383			
Total	1389.9229	5				

1.2.2.1. S. *lacrymans* Forfar exposed to *Trichoderma* isolates grown on minimal.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	183.23	61.076667	16.264633
T. aureoviride	3	144.8079	48.269301	2.7389323

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	246.04293	1	246.04293	25.894396	0.0070381	7.7086497
Within Groups	38.007131	4	9.5017828			
Total	284.05006	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	183.23	61.076667	16.264633
FY Trichoderma	3	78.964309	26.321436	16.270176

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	1811.889	1	1811.889	111.38157	0.000456	7.7086497
Within Groups	65.069618	4	16.267405			
Total	1876.9587	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	183.23	61.076667	16.264633
***T. harzianum* 25**	3	170.88933	56.963109	48.766523

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	25.382039	1	25.382039	0.7806117	0.4268594	7.7086497
Within Groups	130.06231	4	32.515578			
Total	155.44435	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	183.23	61.076667	16.264633
***T. harzianum* 206040**	3	76.09	25.363333	29.543633

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	1913.1633	1	1913.1633	83.529171	0.0007954	7.7086497
Within Groups	91.616533	4	22.904133			
Total	2004.7798	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	183.23	61.076667	16.264633
T. koningii	3	179.84981	59.949938	11.087443

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	1.9042751	1	1.9042751	0.1392417	0.7279691	7.7086497
Within Groups	54.704154	4	13.676038			
Total	56.608429	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	183.23	61.076667	16.264633
T. polysporum	3	153.23836	51.079454	18.068505

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	149.91638	1	149.91638	8.7330428	0.0417542	7.7086497
Within Groups	68.666277	4	17.166569			
Total	218.58266	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	183.23	61.076667	16.264633
T. pseudokoningii	3	189.31033	63.103444	2.3841602

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	6.1617381	1	6.1617381	0.6608189	0.4618845	7.7086497
Within Groups	37.297587	4	9.3243968			
Total	43.459325	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	183.23	61.076667	16.264633
***T. viride* 70**	3	209.44	69.813333	12.627233

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	114.49402	1	114.49402	7.9256919	0.0480591	7.7086497
Within Groups	57.783733	4	14.445933			
Total	172.27775	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	183.23	61.076667	16.264633
***T. viride* 110**	3	144.67526	48.225088	7.0502665

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	247.7446	1	247.7446	21.252041	0.0099557	7.7086497
Within Groups	46.6298	4	11.65745			
Total	294.3744	5				

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	147.80807	1	147.80807	10.787873	0.0303723	7.7086497
Within Groups	54.805267	4	13.701317			
Total	202.61333	5				

1.2.2.2. S. *lacrymans* Forfar exposed to *Trichoderma* isolates grown on sawdust.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	167.95	55.983333	19.312233
T. aureoviride	3	101.29	33.763333	3.0630333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	740.5926	1	740.5926	66.197432	0.0012415	7.7086497
Within Groups	44.750533	4	11.187633			
Total	785.34313	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	167.95	55.983333	19.312233
FY Trichoderma	3	66.06	22.02	0.2757

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	1730.262	1	1730.262	176.66611	0.0001852	7.7086497
Within Groups	39.175867	4	9.7939667			
Total	1769.4379	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	167.95	55.983333	19.312233
***T. harzianum* 25**	3	156.24	52.08	4.8468

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	22.854017	1	22.854017	1.8919645	0.2409831	7.7086497
Within Groups	48.318067	4	12.079517			
Total	71.172083	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	167.95	55.983333	19.312233
***T. harzianum* 206040**	3	49.25	16.416667	8.1657333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	2348.2817	1	2348.2817	170.92107	0.0001976	7.7086497
Within Groups	54.955933	4	13.738983			
Total	2403.2376	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	167.95	55.983333	19.312233
T. koningii	3	150.32	50.106667	19.909733

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	51.802817	1	51.802817	2.6415206	0.1794295	7.7086497
Within Groups	78.443933	4	19.610983			
Total	130.24675	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	167.95	55.983333	19.312233
T. polysporum	3	93.32	31.106667	13.750533

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	928.27282	1	928.27282	56.152156	0.0016964	7.7086497
Within Groups	66.125533	4	16.531383			
Total	994.39835	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	167.95	55.983333	19.312233
T. pseudokoningii	3	172.46	57.486667	3.9602333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	3.3900167	1	3.3900167	0.2913328	0.6180126	7.7086497
Within Groups	46.544933	4	11.636233			
Total	49.93495	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	167.95	55.983333	19.312233
***T. viride* 70**	3	177.58	59.193333	14.221433

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	15.45615	1	15.45615	0.9218288	0.391366	7.7086497
Within Groups	67.067333	4	16.766833			
Total	82.523483	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	167.95	55.983333	19.312233
***T. viride* 110**	3	116.95	38.983333	2.2609333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	433.5	1	433.5	40.188815	0.0031705	7.7086497
Within Groups	43.146333	4	10.786583			
Total	476.64633	5				

1.2.3. S. *lacrymans* H28 exposed to *Trichoderma* isolates grown on malt.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	151.44636	50.482121	2.1319168
T. aureoviride	3	38.970933	12.990311	5.0008755

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	2108.4538	1	2108.4538	591.20011	1.697E-05	7.7086497
Within Groups	14.265585	4	3.5663961			
Total	2122.7194	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	151.44636	50.482121	2.1319168
FY *Trichoderma*	3	33.772039	11.257346	15.381338

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	2307.8745	1	2307.8745	263.55747	8.424E-05	7.7086497
Within Groups	35.026509	4	8.7566272			
Total	2342.901	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	151.44636	50.482121	2.1319168
***T. harzianum* 25**	3	66.376044	22.125348	13.176271

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	1206.1599	1	1206.1599	157.58363	0.0002317	7.7086497
Within Groups	30.616376	4	7.654094			
Total	1236.7763	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	151.44636	50.482121	2.1319168
***T. harzianum* 206040**	3	34.14444	11.38148	4.1171646

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	2293.2902	1	2293.2902	733.96076	1.104E-05	7.7086497
Within Groups	12.498163	4	3.1245407			
Total	2305.7884	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	151.44636	50.482121	2.1319168
T. koningii	3	127.82507	42.608358	0.5052736

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	92.994237	1	92.994237	70.525235	0.0111002	7.7086497
Within Groups	5.2743809	4	1.3185952			
Total	98.268618	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	151.44636	50.482121	2.1319168
T. polysporum	3	42.851602	14.283867	10.347517

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	1965.4704	1	1965.4704	314.99353	5.921E-05	7.7086497
Within Groups	24.958867	4	6.2397168			
Total	1990.4293	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	151.44636	50.482121	2.1319168
T. pseudokoningii	3	148.61127	49.537091	0.0285756

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	1.3396231	1	1.3396231	1.240109	0.3278525	7.7086497
Within Groups	4.3209848	4	1.0802462			
Total	5.6606078	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	151.44636	50.482121	2.1319168
***T. viride* 70**	3	147.85021	49.283404	0.8925861

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	2.1553843	1	2.1553843	1.4252817	0.2984965	7.7086497
Within Groups	6.0490059	4	1.5122515			
Total	8.2043901	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	151.44636	50.482121	2.1319168
***T. viride* 110**	3	100.5154	33.505132	10.223073

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	432.32726	1	432.32726	69.984236	0.0011165	7.7086497
Within Groups	24.709979	4	6.1774949			
Total	457.03724	5				

1.2.3.1. S. *lacrymans* H28 exposed to *Trichoderma* isolates grown on minimal.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	180.54	60.18	2.1943
T. aureoviride	3	129.64	43.213333	16.356433

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	431.80167	1	431.80167	46.553595	0.0024126	7.7086497
Within Groups	37.101467	4	9.2753667			
Total	468.90313	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	180.54	60.18	2.1943
FY Trichoderma	3	107.14	35.713333	23.043233

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	897.92667	1	897.92667	71.158037	0.0011816	7.7086497
Within Groups	50.475067	4	12.618767			
Total	948.40173	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	180.54	60.18	2.1943
***T. harzianum* 25**	3	154.83	51.61	0.0433

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	110.16735	1	110.16735	98.469208	0.0157904	7.7086497
Within Groups	4.4752	4	1.1188			
Total	114.64255	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	180.54	60.18	2.1943
***T. harzianum* 206040**	3	165.24	55.08	27.2761

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	39.015	1	39.015	2.6477415	0.1790262	7.7086497
Within Groups	58.9408	4	14.7352			
Total	97.9558	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	180.54	60.18	2.1943
T. koningii	3	155.81	51.936667	45.078533

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	101.92882	1	101.92882	4.3123633	0.1064289	7.7086497
Within Groups	94.545667	4	23.636417			
Total	196.47448	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	180.54	60.18	2.1943
T. polysporum	3	137.22	45.74	15.1497

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	312.7704	1	312.7704	36.066697	0.0038694	7.7086497
Within Groups	34.688	4	8.672			
Total	347.4584	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	180.54	60.18	2.1943
T. pseudokoningii	3	187.47	62.49	2.0788

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	8.00415	1	8.00415	3.7462966	0.1250172	7.7086497
Within Groups	8.5462	4	2.13655			
Total	16.55035	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	180.54	60.18	2.1943
***T. viride* 70**	3	190.11	63.37	0.0351

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	15.26415	1	15.26415	13.693505	0.0208268	7.7086497
Within Groups	4.4588	4	1.1147			
Total	19.72295	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	180.54	60.18	2.1943
***T. viride* 110**	3	88.24	29.413333	1.7624333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	1419.8817	1	1419.8817	717.704	1.154E-05	7.7086497
Within Groups	7.9134667	4	1.9783667			
Total	1427.7951	5				

1.2.3.2. S. *lacrymans* H28 exposed to *Trichoderma* isolates grown on sawdust.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	167.44	55.813333	9.2745333
T. aureoviride	3	95.100339	31.700113	7.9229339

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	872.1711	1	872.1711	101.43018	0.0005468	7.7086497
Within Groups	34.394935	4	8.5987336			
Total	906.56603	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	167.44	55.813333	9.2745333
FY Trichoderma	3	81.866997	27.288999	4.7946911

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	1220.4565	1	1220.4565	173.49307	0.0001919	7.7086497
Within Groups	28.138449	4	7.0346122			
Total	1248.5949	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	167.44	55.813333	9.2745333
***T. harzianum* 25**	3	138.50079	46.166929	1.6747656

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	139.57968	1	139.57968	25.495637	0.0123454	7.7086497
Within Groups	21.898598	4	5.4746495			
Total	161.47828	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	167.44	55.813333	9.2745333
***T. harzianum* 206040**	3	138.48404	46.161348	8.6628703

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	139.74124	1	139.74124	15.580989	0.0168565	7.7086497
Within Groups	35.874807	4	8.9687018			
Total	175.61605	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	167.44	55.813333	9.2745333
T. koningii	3	140.23389	46.74463	0.4880295

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	123.36206	1	123.36206	25.272475	0.0073481	7.7086497
Within Groups	19.525126	4	4.8812814			
Total	142.88719	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	167.44	55.813333	9.2745333
T. polysporum	3	93.029997	31.009999	13.279241

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	922.80808	1	922.80808	81.831809	0.0012743	7.7086497
Within Groups	45.107549	4	11.276887			
Total	967.91563	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	167.44	55.813333	9.2745333
T. pseudokoningii	3	166.99907	55.666358	7.3811197

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	0.0324025	1	0.0324025	0.0038909	0.9532552	7.7086497
Within Groups	33.311306	4	8.3278265			
Total	33.343709	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	167.44	55.813333	9.2745333
***T. viride* 70**	3	153.45876	51.15292	2.0694731

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	32.579179	1	32.579179	5.7438576	0.0746283	7.7086497
Within Groups	22.688013	4	5.6720032			
Total	55.267192	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	167.44	55.813333	9.2745333
***T. viride* 110**	3	59.954683	19.984894	8.3912935

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	1925.5156	1	1925.5156	217.99326	0.0001225	7.7086497
Within Groups	35.331654	4	8.8329134			
Total	1960.8472	5				

1.2.4. S. *lacrymans* BF050 exposed to *Trichoderma* isolates grown on malt.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	146.84	48.946667	2.5282333
T. aureoviride	3	28.6	9.5333333	15.023233

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	2330.1163	1	2330.1163	265.51813	8.301E-05	7.7086497
Within Groups	35.102933	4	8.7757333			
Total	2365.2192	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	146.84	48.946667	2.5282333
FY Trichoderma	3	28	9.3333333	26.148633

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	2353.8243	1	2353.8243	164.16189	0.0002139	7.7086497
Within Groups	57.353733	4	14.338433			
Total	2411.178	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	146.84	48.946667	2.5282333
***T. harzianum* 25**	3	108.93	36.31	36.8227

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	239.52802	1	239.52802	12.173943	0.025143	7.7086497
Within Groups	78.701867	4	19.675467			
Total	318.22988	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	146.84	48.946667	2.5282333
***T. harzianum* 206040**	3	26.35	8.7833333	6.1700333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	2419.64	1	2419.64	556.34993	1.915E-05	7.7086497
Within Groups	17.396533	4	4.3491333			
Total	2437.0366	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	146.84	48.946667	2.5282333
T. koningii	3	74.49	24.83	57.9856

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	872.42042	1	872.42042	28.833752	0.0058085	7.7086497
Within Groups	121.02767	4	30.256917			
Total	993.44808	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	146.84	48.946667	2.5282333
T. polysporum	3	42.79	14.263333	40.168633

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	1804.4004	1	1804.4004	84.521444	0.0007775	7.7086497
Within Groups	85.393733	4	21.348433			
Total	1889.7942	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	146.84	48.946667	2.5282333
T. pseudokoningii	3	128.42	42.806667	11.737633

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	56.5494	1	56.5494	7.9279305	0.0480397	7.7086497
Within Groups	28.531733	4	7.1329333			
Total	85.081133	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	146.84	48.946667	2.5282333
***T. viride* 70**	3	100.53	33.51	2.3389

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	357.43602	1	357.43602	146.87743	0.0012659	7.7086497
Within Groups	9.7342667	4	2.4335667			
Total	367.17028	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	146.84	48.946667	2.5282333
***T. viride* 110**	3	74	24.666667	2.4990333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	884.2776	1	884.2776	351.7926	4.758E-05	7.7086497
Within Groups	10.054533	4	2.5136333			
Total	894.33213	5				

1.2.4.1. S. *lacrymans* BF050 exposed to *Trichoderma* isolates grown on minimal.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	169.75	56.583333	12.480133
T. aureoviride	3	112.05	37.35	11.2492

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	554.88167	1	554.88167	46.767573	0.002392	7.7086497
Within Groups	47.458667	4	11.864667			
Total	602.34033	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	169.75	56.583333	12.480133
FY Trichoderma	3	166.48	55.493333	9.7442333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	1.78215	1	1.78215	0.160378	0.7092762	7.7086497
Within Groups	44.448733	4	11.112183			
Total	46.230883	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	169.75	56.583333	12.480133
***T. harzianum* 25**	3	178.56	59.52	11.6452

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	12.936017	1	12.936017	1.0724011	0.358901	7.7086497
Within Groups	48.250667	4	12.062667			
Total	61.186683	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	169.75	56.583333	12.480133
***T. harzianum* 206040**	3	114.18	38.06	1.4788

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	514.67082	1	514.67082	73.740708	0.0010103	7.7086497
Within Groups	27.917867	4	6.9794667			
Total	542.58868	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	169.75	56.583333	12.480133
T. koningii	3	189.53	63.176667	0.0558333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	65.208067	1	65.208067	10.403357	0.0321147	7.7086497
Within Groups	25.071933	4	6.2679833			
Total	90.28	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	169.75	56.583333	12.480133
T. polysporum	3	118.81	39.603333	0.4232333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	432.4806	1	432.4806	67.033761	0.0012122	7.7086497
Within Groups	25.806733	4	6.4516833			
Total	458.28733	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	169.75	56.583333	12.480133
T. pseudokoningii	3	190.3	63.433333	0.0136333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	70.38375	1	70.38375	11.267018	0.0283933	7.7086497
Within Groups	24.987533	4	6.2468833			
Total	95.371283	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	169.75	56.583333	12.480133
***T. viride* 70**	3	190.96	63.653333	0.0142333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	74.97735	1	74.97735	12.001785	0.0257154	7.7086497
Within Groups	24.988733	4	6.2471833			
Total	99.966083	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	169.75	56.583333	12.480133
***T. viride* 110**	3	151.38	50.46	1.5376

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	56.242817	1	56.242817	8.0245237	0.0472128	7.7086497
Within Groups	28.035467	4	7.0088667			
Total	84.278283	5				

1.2.4.2. S. *lacrymans* BF050 exposed to *Trichoderma* isolates grown on sawdust.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	171.1	57.033333	7.7232333
T. aureoviride	3	77.47408	25.824693	12.136949

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	1460.9688	1	1460.9688	147.12542	0.0002651	7.7086497
Within Groups	39.720365	4	9.9300912			
Total	1500.6892	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	171.1	57.033333	7.7232333
FY Trichoderma	3	122.60456	40.868186	11.097007

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	391.968	1	391.968	41.653879	0.0029672	7.7086497
Within Groups	37.64048	4	9.4101201			
Total	429.60848	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	171.1	57.033333	7.7232333
***T. harzianum* 25**	3	155.81507	51.938356	4.048317

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	38.938198	1	38.938198	6.6156448	0.061842	7.7086497
Within Groups	23.543101	4	5.8857752			
Total	62.481298	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	171.1	57.033333	7.7232333
***T. harzianum* 206040**	3	102.12959	34.043197	21.656141

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	792.81958	1	792.81958	53.971167	0.0018281	7.7086497
Within Groups	58.75875	4	14.689687			
Total	851.57833	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	171.1	57.033333	7.7232333
T. koningii	3	165.17994	55.05998	1.977085

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	5.8411851	1	5.8411851	1.2043285	0.3340848	7.7086497
Within Groups	19.400637	4	4.8501592			
Total	25.241822	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	171.1	57.033333	7.7232333
T. polysporum	3	105.21509	35.071698	11.376227

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	723.47015	1	723.47015	75.758176	0.0011959	7.7086497
Within Groups	38.198921	4	9.5497303			
Total	761.66908	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	171.1	57.033333	7.7232333
T. pseudokoningii	3	169.34908	56.449692	12.156932

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	0.5109554	1	0.5109554	0.0514035	0.8317541	7.7086497
Within Groups	39.760331	4	9.9400827			
Total	40.271286	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	171.1	57.033333	7.7232333
***T. viride* 70**	3	152.6155	50.871832	1.0893414

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	56.946144	1	56.946144	12.923838	0.0228602	7.7086497
Within Groups	17.625149	4	4.4062874			
Total	74.571293	5				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	171.1	57.033333	7.7232333
***T. viride* 110**	3	135.95036	45.316786	2.406303

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	205.91624	1	205.91624	40.656597	0.0031034	7.7086497
Within Groups	20.259073	4	5.0647682			
Total	226.17531	5				

APPENDIX B

Appendix B

B 1.1. Phosphate buffered saline (PBS) containing Proteinase inhibitors

One tablet of PBS (Sigma, Dorset, UK) was dissolved in 200 µl of u-p dH_2O and the following proteinase inhibitors added :

1mM phenylmethylsulfonylfluoride

1mM benzamidine

1mM ethylenediaminetetraacetic acid

Stored at 4°C.

B 1.2. Resolving gel buffer

Tris-HCl	181.5g
SDS	4g

pH to 8.9 with HCL at room temperature and make up to 1 litre with u-p dH_2O and store at 4°C.

B 1.3. Stacking Gel Buffer

Tris-HCl	59.0g
SDS	4.0g

pH to 6.7 with HCL at room temperature and make up to 1 litre with u-p dH_2O and store at 4°C.

B 1.4. 5 X Electrode Buffer

Tris-HCl	15.15g
Glycine	72g
SDS	5g
Up-dH_20	1l

Stored at 4°C and made to 1 X strength before use

B 1.5. Laemmli Sample Buffer

4% SDS

20% glycerol

10% 2-mercaptoethanol

0.004% bromophenol blue

0.125 M Tris-HCL, pH 6.8

Aliquoted into 1ml and stored at -20°C.

B 2.1. Repetition of SDS-PAGE of *S. lacrymans* cultures exposed to *Trichoderma* VOCs.

The gel and dendogram for *S. lacrymans* H28 is shown in figure B2.1a and B2.1b respectively. This is shown as an example of the reproducibility when the experiment in chapter 4 was repeated. As can be seen from figure B2.1a the same proteins are affected by the *Trichoderma* VOCs as in chapter 4 (Fig. 4.6a, page 104). The Repeat dendogram analysis shows the same percentage similarity as the results in chapter 4 (Fig. 4.6b, page 105) with *T. pseudokoningii* being 100% similar, *T. viride* and *T. aureoviride* are both 80% similar when compared with the controls and *T. aureoviride* and *T. viride* are 94% similar to one another.

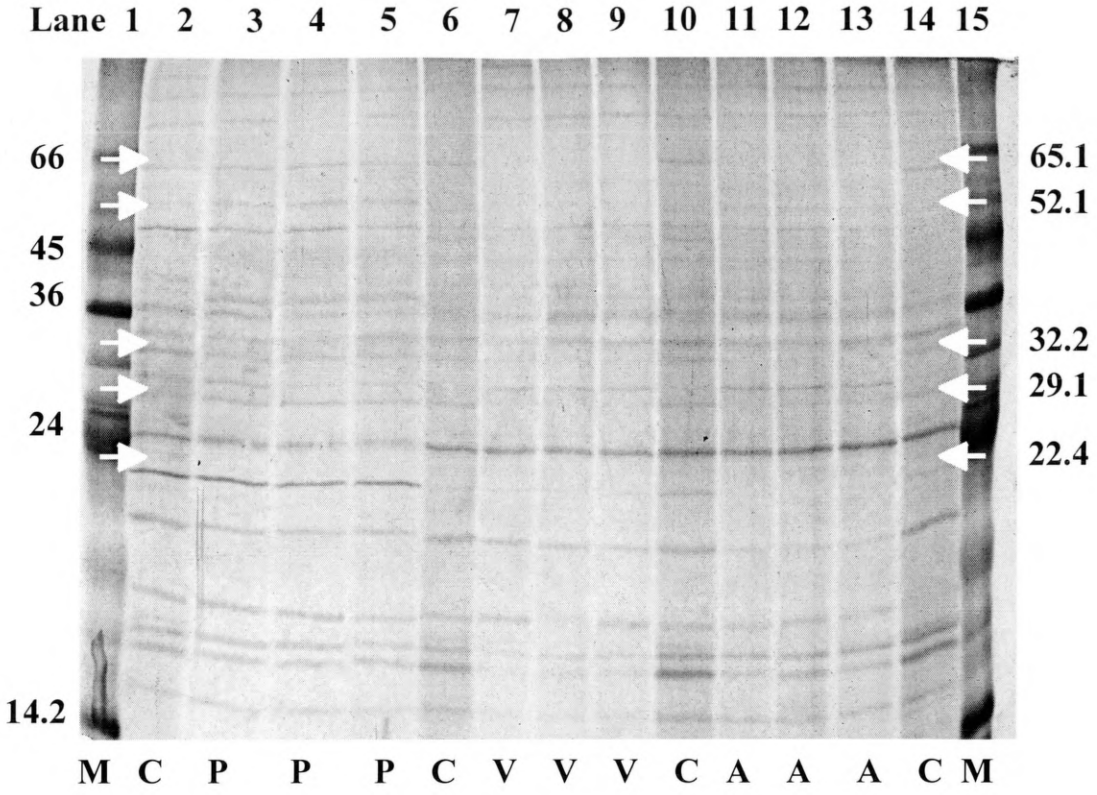

Figure B2.1a. Protein profile of repeat *S. lacrymans* H28; lanes 2,6,10 and 14 are the control culture which was not exposed to any antagonist; lanes 3-5 were grown in the presence of *T. pseudokoningii*; lanes 7-9 were grown in the presence of *T. viride* and lanes 11-13 were grown in the presence of *T. aureoviride*. Lanes 1 and 15 are molecular weight markers. The proteins affected by the *Trichoderma* VOCs can be seen down the right hand side of the gel with weights of the markers in kDa on the left.

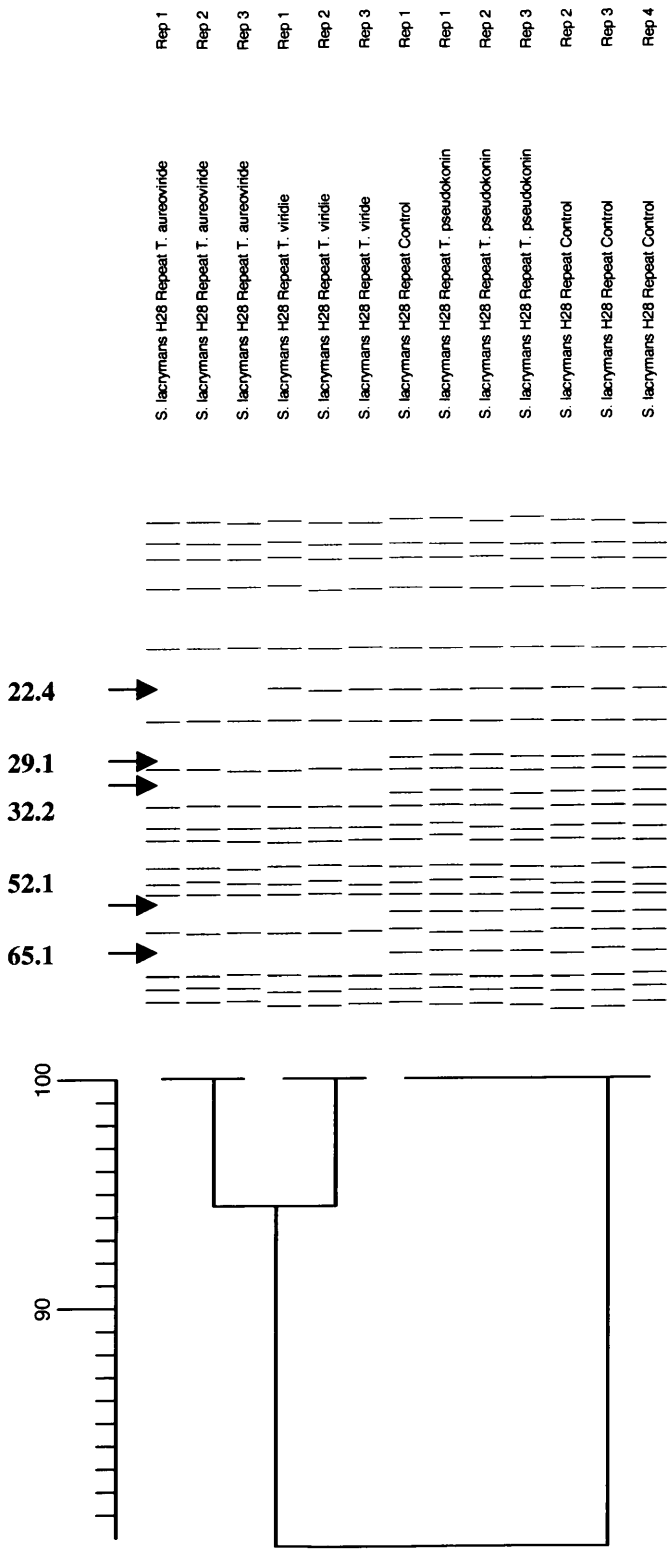

Figure B2.1b. Gelcompar generated dendogram of the bands produced by repeat *S. lacrymans* H28. The scale bar represents % similarity.

The published paper cited below has been removed from the e-thesis due to copyright restrictions:

Humphris, S.N., Bruce, A., Buultjens, E. and Wheatley, R.E. (2002). The effects of volatile microbial secondary metabolites on protein synthesis in *Serpula lacrymans*. In, *FEMS Microbiology Letters*, 210, pp.215-219. DOI: 10.1016/S0378-1097(02)00604-3

IRG/WP 00-10332

THE INTERANTIONAL RESEARCH GROUP ON WOOD PRESERVATION

SECTION 1 BIOLOGY

The Effect of *Trichoderma* Volatiles on the Growth and Enzyme Production of *Serpula Lacrymans*

Sonia N. Humphris,* Alan Bruce* and Ron E. Wheatley**.

* Scottish Institute for Wood Technology, School of Science and Engineering, University of Abertay Dundee, Dundee. DD1 1HG, Scotland, U.K.

** Soil Plant Dynamics Unit, Division of Plant, Soils and Environment, Scottish Crop Research Institute, Invergowrie, Dundee. DD2 5DA, Scotland, U.K.

Paper prepared for the 31st Annual Meeting
Kona, Hawaii
May 2000

IRG Secretariat
SE-100 44 STOCKHOLM
Sweden

The Effect of *Trichoderma* Volatiles on the Growth and Enzyme Production of *Serpula Lacrymans*

Sonia N. Humphris,* Alan Bruce* and Ron E. Wheatley**.

* Scottish Institute for Wood Technology, School of Science and Engineering, University of Abertay Dundee, Dundee. DD1 1HG, Scotland, U.K.
** Soil Plant Dynamics Unit, Division of Plant, Soils and Environment, Scottish Crop Research Institute, Invergowrie, Dundee. DD2 5DA, Scotland, U.K.

ABSTRACT

Although various authors have reported the biological control of *Serpula lacrymans* by *Trichoderma* spp. the mode of antagonism employed by *Trichoderma* is not yet clear. The work presented here concentrates on the production of anti-fungal volatiles for inhibition of *S. lacrymans* growth. Volatile mediated interactions were examined between four *S. lacrymans* isolates and a range of nine known *Trichoderma* antagonists. Interactions were evaluated on two agar media and Scots pine sawdust. Results indicated that *S. lacrymans* isolates were significantly inhibited by volatile organic compounds (VOCs) produced by *Trichoderma* isolates, with some *Trichoderma* isolates being more potent producers of inhibitory VOCs than others. The production of VOCs was influenced by the nutrient composition of the *Trichoderma* growth media with generally greater inhibition being produced when *Trichoderma* isolates were cultured on nutrient rich media. Results from cultures grown on sawdust gave greater inhibition than low nutrient medium but less than when cultures were grown on malt extract agar. The effect of VOCs on fungal enzyme production was also examined by testing tyrosinase, cellulase and peroxidase. Results indicated that production of cellulase was not affected by *Trichoderma* volatiles and that tyrosinase was not released by any of the *S. lacrymans* isolates. Inhibition of growth of the *S. lacrymans* isolates by *Trichoderma* volatiles increased peroxidase activity. The paper discusses the potential role of VOC production in the biological control of dry rot.

Keywords : Biological control; *Trichoderma* spp; *Serpula lacrymans*; VOC production.

INTRODUCTION

Dry rot currently requires radical treatment with chemicals and is often extremely expensive. The need for such extreme treatment methods is becoming increasingly difficult to justify on health and safety grounds and new methods of treatment have been considered, one of which is biological control. Palfreyman *et al.*, (1995) have shown that antagonism does exist between *S. lacrymans* and various *Trichoderma* spp. during malt extract confrontations. Score & Palfreyman (1994a,b) examined a range of interactions between *S. lacrymans* and *Trichoderma* isolates on several different media and a small-scale wood block system. A number of *Trichoderma* isolates were identified which may be effective in the remedial treatment of dry rot. It has also previously been shown that *Trichoderma* spp. can prevent or reduce weight loss in wood blocks that are infected with *Serpula lacrymans* in contact with soil (Doi & Yamada 1991, 1992). Score (1998) found that *Trichoderma* could be used to protect wood from decay and prevent the spread of *Serpula lacrymans* during medium-scale wood block experiments and field trial experiments. The *Trichoderma*, however, could not kill an existing *S. lacrymans* colony in the wood block tests. The exact mode of antagonism that *Trichoderma* employs to control *S. lacrymans* is not yet fully understood. Srinivasan (1992) & Score (1998) have both shown the importance of the nutrient composition on the effectiveness of biological control by *Trichoderma* isolates. To realise the full potential of *Trichoderma* as bioprotection/biocontrol agents their mode of antagonism must be determined. Through years of extensive investigation it has been determined that *Trichoderma* species antagonise other fungi through a variety of active mechanisms, one of these being the production of volatile organic compounds (VOCs). Srinivasan *et al.*, (1992) demonstrated the importance of nutrient composition on volatile inhibition by *Trichoderma* isolates with greater levels of inhibition of wood decay fungi being produced by *Trichoderma* isolates when grown on nutrient rich media.

The effects of VOCs on fungal metabolism can also be examined by monitoring the production of enzymes. Tyrosinase production is thought to be linked to pigmentation (Jolivet *et al.*, 1998; Flurkey *et al.*, 1995) where the enzyme catalyses two different reactions, firstly the hydroxylation of monophenols to o-diphenols and the oxidation of o-phenols to o-quinones, which in turn are polymerised to pigments (Espin *et al.*, 1999). Tyrosinase production is also linked to morphogenisis (Griffith *et al.*, 1994). White and Boddy, (1992) found that phenol-oxidasing enzymes are correlated to the production of aerial mycelium in *P. radiata*. Peroxidase enzymes have been implicated in the production of highly toxic compounds that are antifungal in nature and also in the formation of different mycelial formations and pigment production (Score 1998). Although there are no reports of VOCs that inhibit these enzymes, Score *et al.*, (1997) examined whether three enzymes (laccase, tyrosinase and peroxidase) were produced during basidiomycete confrontations by performing a number of pairings between two brown rot fungi, *Serpula lacrymans* and *Coniophora puteana* and *Trichoderma* spp. or *Scytalidium* FY. Peroxidase release was detected

during all interspecific interactions. Tyrosinase release was observed most frequently during interspecies interaction between *Trichoderma* spp.

The aim of this study was to examine whether VOCs produced by a selection of *Trichoderma* cultures would inhibit the growth of four *Serpula lacrymans* isolates on various media. The effects of these VOCs on the production of tyrosinase, peroxidase and cellulase by *S. lacrymans* isolates was also screened.

MATERIALS AND METHODS

Volatile Inhibition of *Serpula lacrymans*

The *Trichoderma* isolates used in this experiment were *T. aureoviride* (Rifai) IMI 91968, *T. harzianum* (Rifai) Scottish Institute for Wood Technology Culture collection (SIWT) 25, *T. harzianum* (Rifai) IMI 206040, *T. polysporum* (Link ex Pers.) IMI 206039, *T. psuedokoningii* (Rifai) SIWT 64, *T. koningii* (Rifai) IMI 54693, *T. viride* (Pers ex S.F. Gray) SIWT 70, *T.viride* (Pers ex S.F. Gray) SIWT 110 and a culture of *Trichoderma* isolated form Binab FYT pellets (Bioinnovation Ab Binab, Sigtuna, Sweden) containing *T. harzianum* IMI 25, *T. polysporum* IMI 206039and *Scytalidium FY (Pesante)* ATCC 16675. Petri dishes containing 3% malt extract agar were inoculated in the centre with a single plug (5mm in diameter) of each culture of *Trichoderma*, removed form the margins of actively growing colonies. Separate plates of 5% malt extract agar were inoculated with four isolates of *Serpula lacrymans* (Schum. Ex Fr) S.F. Gray: *S. lacrymans* FPRL 12C, *S. lacrymans* BF050, *S. lacrymans* H28 (a himalayan isolate) and *S. lacrymans* Forfar (isolated from a building in Scotland). After removal of the lids, plates inoculated with the *S. lacrymans* were covered with a semipermeable polyvinyl chloride cling film membrane and placed on top of the plates inoculated with the *S. lacrymans*. This experiment was also repeated but with the *Trichoderma* grown on a minimal media and Scot's pine sawdust instead of malt extract. The minimal media had a carbon : nitrogen ratio similar to that typically found in wood and was based on Hutterman and Volger (1973) consisting of 5g D-glucose; 1g KH2PO4; 0.5g KCL; 0.3g MgSO4; 0.01g FeSo4; 0.008g Mn(CH3COO)2.4H2O; 0.002g Zn(No3)2 6H2O; 0.05g Ca(No3)2.4H2O; 0.002g CuSo4; 0.008g NH4No3; 0.013g L-asparagine (anhydrous) with 1.5% purified agar per litre. Autoclaved Scot's pine sawdust (17% w/v moisture content) was wetted to bring the moisture content up to 120% and was then inoculated with the appropriate *Trichoderma* and incubated for one week before the plates of *S. lacrymans* were placed on top. This was to allow the *Trichoderma* time to colonise the sawdust. Controls were set up on three media types in an identical fashion except they contained no *Trichoderma* inocula on the bottom plate. All cultures were incubated at 21°C until the growth of the *S. lacrymans* on the control plates had reached the edge of the plate (usually 7 days). Three replicates were set up for all tests and controls. Growth of the *S. lacrymans* was measured using a Digital Image Analysis System (Delta-T Devices, U.K.), that recorded growth as an

area in cm 2. Inhibition of growth of the *S. lacrymans* was recorded as the difference in mean growth area of the *S. lacrymans* in the presence or absence of the *Trichoderma* cultures as a percentage of the controls.

Enzyme Production

Identical tests were carried out as described above with the *Trichoderma* on 3% malt extract and the *S. lacrymans* on 5% malt extract agar. Enzyme tests were carried out as described by White & Boddy (1992). After the *S. lacrymans* cultures had been exposed to the *Trichoderma* isolates, the following enzyme tests were undertaken :

1. Tyrosinase – 5mls of 1.08% (w/v) p-cresol in ethanol was added to the *S. lacrymans* culture, plates were left at room temperature for 24h to allow any colour reaction to develop. A red colour reaction indicated tyrosinase activity.

2. Peroxidase - 5mls of 1.26% (w/v) pyragallol in distilled water, added to 0.4% hydrogen peroxide was used to assess peroxidase production. The plates were left at room temperature for 24h to allow any colour reaction to develop. A brown colour reaction indicated peroxidase activity

3. Cellulase - Cellulose azure was sprinkled over the 5% malt extract plates before the *S. lacrymans* was inoculated and inverted over the plates of *Trichoderma*. After the plates had incubated in contact with the *Trichoderma* VOCs for 7 days they were checked for release of cellulose azure dye into the media. Release of a red colouration indicated cellulase activity.

RESULTS

Volatile Inhibition of *Serpula lacrymans*

The levels of inhibition of growth of the *S. lacrymans* isolates by VOCs from the nine *Trichoderma* isolates are shown in Figure 1. The results show that levels of inhibition are very variable and are dependent on the *Trichoderma* isolate; the sensitivity of the *S. lacrymans* strains and also the media type. It is clear from the results that volatile inhibition of all four *S. lacrymans* cultures was significantly greater on the malt extract agar compared with the minimal media and the Scot's pine sawdust. An interesting anomaly was *T. viride* T110 that gave higher levels of inhibition when grown on sawdust and minimal media rather than malt for *S. lacrymans* H28 and FPRL 12C. Levels of inhibition tended to be slightly greater when the *Trichoderma* cultures were grown on the sawdust media when compared with the minimal media. On all three media types all *S. lacrymans* isolates showed greatest inhibition by VOCs from *T. polysporum* IMI 206039, *T. aureoviride* IMI 91968, *T. harzianum* IMI 206040 and FY *Trichoderma*. The mycelium of the *S. lacrymans* regularly produced a yellow pigmentation when exposed to the VOCs produced by these isolates. *S.*

lacrymans FPRL 12C and Forfar were inhibited slightly more by the VOCs produced by these *Trichoderma* cultures than *S. lacrymans* H28 and BF050. On the minimal media and sawdust media *S. lacrymans* BF050 was generally less susceptible to the volatiles produced by *Trichoderma* than the other three *S. lacrymans* isolates.

Enzyme Production

The effects of VOCs produced by the *Trichoderma* isolates on the enzyme production of the *S. lacrymans* cultures can be seen in Table 1. The results of these tests show that cellulase production by *S. lacrymans* was unaffected by the volatiles produced by *Trichoderma* on malt extract agar. Cellulase was produced by all cultures of *S. lacrymans* including controls and cultures that were inhibited by the *Trichoderma* VOCs. Tyrosinase production was not detected in any of the cultures of *S. lacrymans*, including the cultures that were inhibited by the *Trichoderma* isolates. Peroxidase production was detected in all the cultures of *S. lacrymans* including the control plates and plates that had been exposed to inhibitory *Trichoderma*. Isolates which displayed less inhibition of growth such as *T. koningii* IMI 54693 and *T. psuedokoningii* T64, showed peroxidase production only around the core of the *S. lacrymans* and not elsewhere on the mycelium. *Serpula* isolates however displayed a brown colour (positive for peroxidase production) around the core and spreading out over the mycelium of the cultures exposed to *T. polysporum IMI 206039*, *T. aureoviride* IMI 91968, *T. harzianum* IMI 206040 and FY *Trichoderma*. These *Trichoderma* isolates displayed the greatest inhibition of *S. lacrymans* cultures on malt extract. *S. lacrymans* FPRL 12Cand *S. lacrymans* H28 that had been exposed to the volatiles produced by *T. viride* T110 on malt extract agar only showed peroxidase production around the core, the other two *S. lacrymans* isolates, BF050 and Forfar exposed to *T. viride* T110 however, showed peroxidase production around the core and over the mycelium of the culture. *S. lacrymans* 12C and H28 when exposed to *T. harzianum* 25 also showed peroxidase production around the core and over the mycelium of the culture, whereas BF050 and Forfar exposed to the VOCs of T. harzianum 25 only showed peroxidase around the core.

DISCUSSION

Srinivasan (1992), Score & Palfreyman (1994) and Wheatley *et al.*, (1997) have all shown the importance of nutrient composition on the effectiveness of biological control by *Trichoderma* isolates. The results presented here demonstrate that the composition of media has a major effect on the volatile inhibition of *S. lacrymans* by *Trichoderma* species and that different strains of *Trichoderma* give varying levels of inhibition of *S. lacrymans*. This shows that the VOCs produced are dependent on both the media type and the *Trichoderma* isolate. Over all three media the same *Trichoderma* isolates consistently produced the greatest levels of inhibition, namely *T. polysporum* IMI 206039, *T. aureoviride* IMI 91968, *T. harzianum* IMI 206040 and FY *Trichoderma*. However, the *S. lacrymans* isolates also showed different

sensitivities to the VOCs produced by these *Trichoderma* isolates. For example on the malt extract media *S. lacrymans* Forfar was inhibited by 91% by *T. aureoviride* IMI 91968 whereas *S. lacrymans* H28 was inhibited by 74%. Interestingly *T. viride* T110 gave higher levels of inhibition when grown on sawdust rather than malt media for *S. lacrymans* H28 and FPRL 12C. This could be because T. viride T110 is producing different volatiles when grown on the sawdust or producing certain inhibitory volatiles in greater amounts. Work is continuing to try and identify the active VOCs that are responsible for the inhibition of the *S. lacrymans*, as such compounds could be used in chemical fumigation of wooden structures or as a basis for the selection of *Trichoderma* strains for biological control purposes. Previous work by Wheatley *et al.*, (1997) identified five VOCs acetone, 2methyl-1-butanol, heptanal, octanal and decanal produced by *Trichoderma*. These VOCs were implicated in the inhibition of the four wood decay fungi, *Neolentinus lepideus* (Fr:Fr.) Redhead and Ginns (FPRL 7G), *Postia placenta* (Fr) M. Lars et Lomb (FPRL 280), *Gloeophyllum trabeum* (Pers:Fr) Murr. (FPRL 108 N) and *Trametes versicolor* (L.:Fr.) Pilat. (FPRL 28G). Humphris *et al.*, (1999) tested four of these five compounds, acetone, 2methyl-1-butanol, heptanal and octanal against the four wood decay fungi and found that the two aldehydes, heptanal and octanal were effective in inhibiting all four fungi. Future work will determine if the same volatiles inhibit *S. lacrymans* or if a different selection of compounds is responsible.

It may be possible to develop a biological control strategy for *S. lacrymans* by either enhancing or inhibiting the production of specific enzymes by the decay fungus. In this experiment the production of three enzymes, tyrosinase, peroxidase and cellulase was examined. VOC production by *Trichoderma* did not stimulate the production of tyrosinase by the *S. lacrymans* isolates with no colour reaction appearing in any of the combinations of *Trichoderma* and *S. lacrymans* isolates. The function of tyrosinase has been linked to morphogenesis and pigmentation (Jolivet *et al.*, 1998; Flurkey *et al.*, 1995; Griffith *et al.*, 1994). The results of this experiment indicate that tyrosinase was not produced by *S. lacrymans* in either control plates or plates exposed to the VOCs produced by *Trichoderma*. Since yellow pigmentation was often detected in plates of *S. lacrymans* that had been inhibited it is unlikely that tyrosinase plays a role in this pigment production. Score *et al.*, (1997) also concluded that tyrosinase was not involved in pigment production by *S. lacrymans* after he found that the production of tyrosinase by *S. lacrymans* was inhibited in confrontation experiments with *Trichoderma* species on malt extract where yellow and brown pigmentation was seen. Zare-Mavain & Shearer (1988) demonstrated that tyrosinase production was dependent on the medium used, with only 10% of freshwater lignicolous fungi producing tyrosinase on malt extract media whereas 45% of the strains of fungi produced this enzyme on cornmeal agar. The use of malt extract agar may therefore account for the level of production of this enzyme by *S. lacrymans* in this study. Peroxidase enzymes have been implicated in the production of highly toxic compounds that are antifungal in nature and also in the formation of different types of mycelia and pigment production (Score 1998). Increased peroxidase production was detected in all *S. lacrymans* cultures that were inhibited by the VOCs from *T. polysporum* IMI 206039, *T. aureoviride* IMI 91968, *T. harzianum* IMI 206040 and

FY *Trichoderma* on malt extract agar and in *S. lacrymans* BF050 and Forfar when exposed to *T. viride* T110 on malt extract agar in *S. lacrymans* 12C and H28 when exposed to *T. harzianum* 25. All these cultures displayed yellow pigmentation and this suggests that increased peroxidase production is linked to increased pigmentation associated with a stress reaction in the fungus. Score & Palfreyman (1994) also reported that interactions between *S. lacrymans* and *Trichoderma* isolates induced a yellow and brown pigmentation in *S. lacrymans*. Wood degradation by brown rot fungi is reported to include enzymatic and non-enzymaticprocesses (Highley 1987). Ritschkoff *et al.*, (1992) reported that carbohydrate degrading enzymes were produced by *Gloeophyllum trabeum* immediately after its inoculation on sawdust media indicating that these enzymes are involved in the degradation of the amorphous, easily degradable parts of cellulose and a non-enzymatic process is involved in the degradation of crystalline cellulose. This study has shown that *S. lacrymans* produces cellulase after only 7 days incubation and that cellulase was produced continuously by the *S. lacrymans* isolates and was not affected by the volatiles produced by *Trichoderma*. Therefore, this suggests that *S. lacrymans* would still be capable of degrading the amorphous parts of cellulose even if growth was inhibited by *Trichoderma* VOCs.

This paper has shown that VOCs produced by *Trichoderma* have an inhibitory effect on the growth of *Serpula lacrymans* and have influenced the production of one of the enzymes, namely peroxidase. Inhibition of *Serpula* by *Trichoderma* volatiles is however dependent on media type and *Trichoderma* isolate as well as sensitivity of the *Serpula* isolate. Future work on the identification and mechanisms of action of such active VOCs may provide an alternative strategy for the remedial treatment of dry rot.

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Legend for Figure1.
Fig. 1. - Growth inhibition (%) of the four *Serpula lacrymans* isolates exposed to the VOCs from nine *Trichoderma* isolates grown on malt extract, minimal media and Scots pine sawdust. Bars represent standard deviations.

ACKNOWLEDGEMENTS

The project was funded by a grant under the EU CRAFT project FAS2 9073 - Biological Control as part of an environmentally friendly future for the eradication of dry rot from buildings initiative. The authors would like to thank Dr Gordon Low of the Dry Rot Research Group for supplying the *S.lacrymans* isolates.

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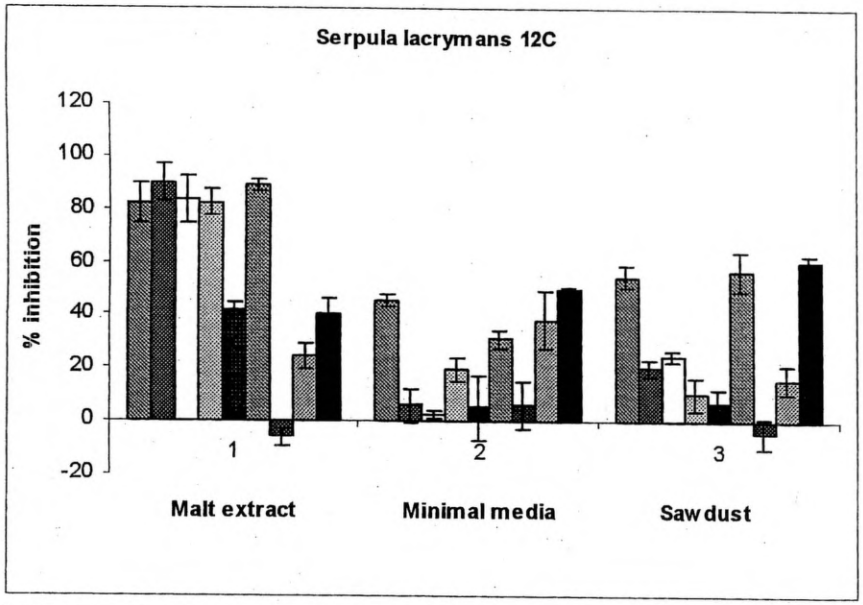
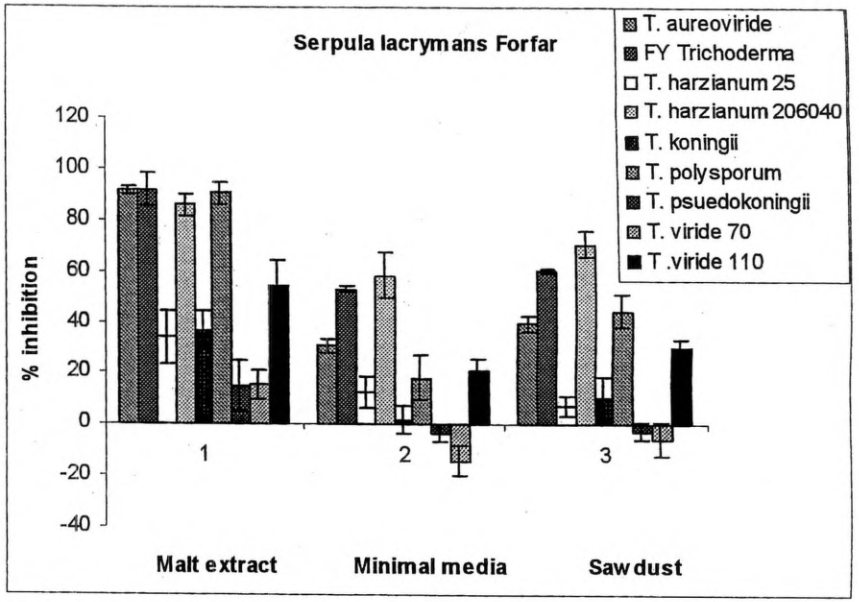
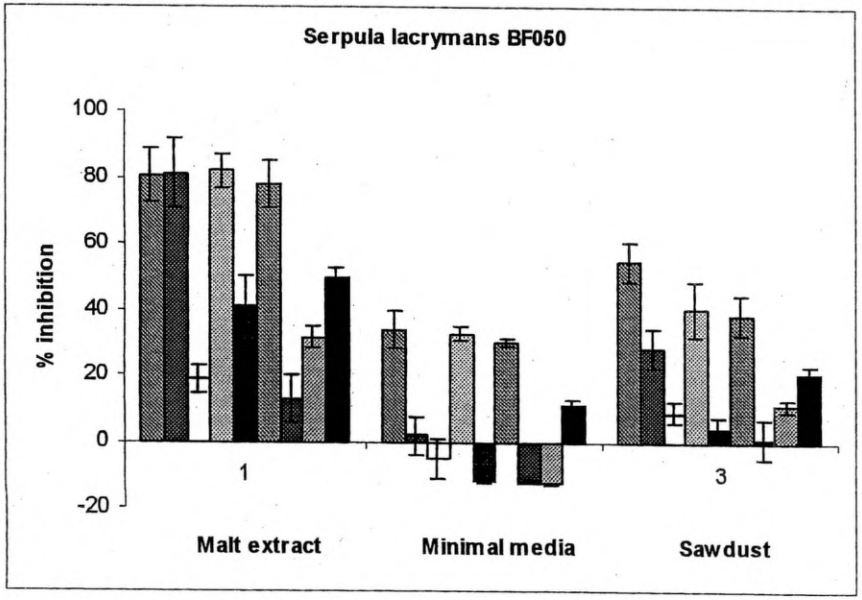

Serpula lacrymans 12C
% inhibition
120
100
80
60
40
20
0
-20
1
2
3
Malt extract
Minimal media
Sawdust


Serpula lacrymans Forfar
T. aureoviride
FY Trichoderma
T. harzianum 25
T. harzianum 206040
T. koningii
T. polysporum
T. psuedokoningii
T. viride 70
T .viride 110
% inhibition
120
100
80
60
40
20
0
-20
-40
1
2
3
Malt extract
Minimal media
Sawdust


Serpula lacrymans BF050
% inhibition
100
80
60
40
20
0
-20
1
3
Malt extract
Minimal media
Sawdust

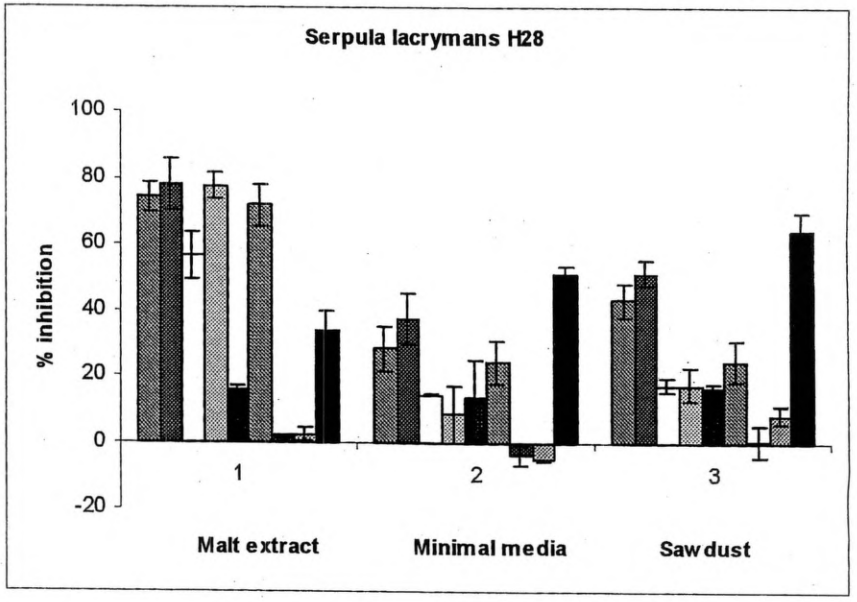

Serpula lacrymans H28
% inhibition
100
80
60
40
20
0
-20
1
2
3
Malt extract
Minimal media
Sawdust

Table 1. Enzymes detected in cultures of *S. lacrymans* exposed to VOCs from *Trichoderma*

Trichoderma	**Cellulase**				**Peroxidase**				**Tyrosinase**			
	12C	Forfar	BF050	H28	12C	Forfar	BF050	H28	12C	Forfar	BF050	H28
Control	X	X	X	X	X	X	X	X	N	N	N	N
T. aureoviride 91968	X	X	X	X	XX	XX	XX	XX	N	N	N	N
FY *Trichoderma*	X	X	X	X	XX	XX	XX	XX	N	N	N	N
T. harzianum T 25	X	X	X	X	XX	X	X	XX	N	N	N	N
T. harzianum IMI 206040	X	X	X	X	XX	XX	XX	XX	N	N	N	N
T. koningii IMI54693	X	X	X	X	X	X	X	X	N	N	N	N
T. polysporum IMI 206039	X	X	X	X	XX	XX	XX	XX	N	N	N	N
T. psuedokoningii T 64	X	X	X	X	X	X	X	X	N	N	N	N
T. viride T 70	X	X	X	X	X	X	X	X	N	N	N	N
T. viride T 110	X	X	X	X	X	XX	XX	X	N	N	N	N

Key:

N – No enzyme release detected
X – enzyme release detected
XX – increased enzyme production